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<b>13. ABSTRACT (Maximum 200 Words)</b> Glutamic acid is both a neurotransmitter in the brain as well as a major neurotoxin, killing nerve cells during trauma and ischemia. Glutamate kills cells through receptor mediated excitotoxicity or via an oxidative stress pathway called oxidative glutamate toxicity. During the past year our laboratory has been studying the pathways which lead to glutamate induced cell death by the oxidative pathway. Using an expression cloning strategy, we have identified a protein, the translation initiation factor $\alpha$ IF2 $\alpha$ , which acts as a central switch that determines whether cells live or die in response to oxidative stress. This appears to be done by the ability of eIF2 $\alpha$ to control the rate of glutathione synthesis by the translational regulation of the rate limiting enzyme in glutathione synthesis. An excellent model for glutamate excitotoxicity was also developed, and it was shown that oxidative glutamate toxicity is a component of the excitotoxicity cascade, a result which explains a great deal of confusing data on glutamate neurotoxicity. Finally, using Bax knock-out mice, we have ruled out the role of this classical death gene in the programmed cell death caused by both forms of glutamate toxicity.				
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## INTRODUCTION

Oxidative stress can be initiated by inhibitors of the mitochondrial electron transport chain, such as nitrogen mustards, cyanide and arsenic derivatives, or by endogenous toxins like glutamic acid. It is likely that there are defined biochemical pathways which cells have evolved for becoming more resistant to oxidative stress. If these are identified, it may be possible to artificially induce them or otherwise modify cellular metabolism such that significant protection could be obtained from the toxic agent. Using a clonal cell model system for oxidative stress (oxidative glutamate toxicity in a hippocampal cell line, HT22), we have isolated clones which are very resistant to various toxic insults, including nitrogen mustards and arsenite. Eight proteins have been identified which may mediate this form of toxicity and two proteins have been chosen for further study: eukaryotic initiation factor 2 $\alpha$  (eIF-2 $\alpha$ ), and soluble guanylate cyclase (sGC). We plan to study these proteins in detail. The additional goals of the project are to further identify the components of the programmed cell death pathway initiated by oxidative and to characterize compounds or conditions which block this pathway. This year's work concentrated upon the role of the translation initiation factor eIF2 $\alpha$  in the oxidative stress cell death pathway. We also examined the potential role of the cell death protein Bax in both oxidative stress and excitotoxicity elicited by glutamate and we were able to show that oxidative glutamate toxicity is a component of the excitotoxicity cascade. Together these results lead to a better understanding of the biochemical mechanisms of oxidative stress in neurotoxicity.

## BODY

As outlined above, three areas of work have been completed during the last year. These include examining the role of the cell death (apoptosis) gene in oxidative glutamate toxicity and excitotoxicity, the role of eIF2 $\alpha$  in oxidative stress, and the involvement of oxidative glutamate toxicity in excitotoxicity. The results from each of these studies will be outlined below and the completed manuscripts with all of the data and details are affixed to the appendix.

### A. Bax

Bax is a required protein for most forms of apoptotic programmed cell death. It is thought to regulate the permeability of mitochondria to proteins which mediate the activation of caspases and other components of apoptosis. Nerve cell death from both oxidative glutamate toxicity and excitotoxicity share a few characteristics with classical apoptosis, but no one has ever examined the role of Bax, the major player in the classical apoptosis pathway, in these pathways. This is critical information, for if Bax is not involved, then another form of programmed cell death than classical apoptosis must take place during glutamate neurotoxicity. To approach this issue, we used mice which lack the Bax gene, so-called Bax knock-out mice. These mice are developmentally abnormal and die before birth, but it is possible to obtain brain nerve cells for primary cultures from E14 embryos and study their response to glutamate. When this was done, it was shown that cortical neurons died equally well when they were isolated from homozygous or heterozygous Bax deletions and from wild type mice. In contrast, the rate of spontaneous cell death when cells are initially placed in culture, due to growth factor withdrawal, was greatly retarded in the cell cultures from Bax knock-out animals. These results clearly show that Bax is not involved in either oxidative glutamate toxicity or excitotoxicity caused by a brief exposure to low concentrations of glutamate. They therefore strongly suggest that a unique form of nerve cell death is involved in glutamate toxicity, which certainly utilizes a distinct set of molecular components. The manuscript detailing these results is in the appendix.

### B. eIF2 $\alpha$ and oxidative stress

Although programmed cell death (PCD) is a widely used mechanism for sculpturing the developing nervous system, its inappropriate activation leads to premature nerve cell death in neuropathological disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). These forms of nerve cell death as well as those caused by a wide variety of neurotoxins are thought to be linked to oxidative stress, for antioxidant systems are upregulated and there is extensive evidence for excessive lipid and protein peroxidation. Associated with oxidative stress, there is usually an early and highly specific decrease in neuronal glutathione content. In the substantia nigra of PD patients, this loss may precede the death of dopaminergic neurons. In



addition, the inhibition of  $\gamma$ -glutamyl-cysteine synthetase (GCS), the rate-limiting step in GSH synthesis, results in the selective degeneration of dopaminergic neurons, and also potentiates the toxicity of 6 hydroxydopamine, MPTP and MPP<sup>+</sup>. These data suggest that GSH and oxidative stress play pivotal roles in neurotoxicity and in the pathogenesis of AD and PD.

There are several ways in which the concentration of intracellular GSH and the oxidative burden of cells can be regulated. One of these is through extracellular glutamate. Although glutamate is generally thought of as both a neurotransmitter and an excitotoxin, extracellular glutamate can also kill neurons through a non-receptor mediated pathway which involves the glutamate-cystine antiporter, system Xc<sup>-</sup>. Under normal circumstances the concentration of extracellular cystine is high relative to intracellular cystine, and cystine is imported via the Xc<sup>-</sup> antiporter in exchange for intracellular glutamate. Cystine is ultimately converted to cysteine and utilized for protein synthesis and to make the antioxidant glutathione (GSH). However, when there is a high concentration of extracellular glutamate, the exchange of glutamate for cystine is inhibited, and the cell becomes depleted of cysteine and GSH, resulting in severe oxidative stress. The cell eventually dies via a series of events which include the depletion of GSH, a requirement for macromolecular synthesis and caspase activity, lipoxigenase (LOX) activation, soluble guanylate cyclase activation, reactive oxygen species (ROS) accumulation, and finally Ca<sup>2+</sup>-influx.

Programmed cell death caused by oxidative glutamate toxicity has characteristics of both apoptosis and necrosis, and has been well studied in primary neuronal cell cultures, neuronal cell lines, tissue slices, and in the immortalized mouse hippocampal cell line, HT22. HT22 cells lack ionotropic glutamate receptors but die within 24 hours after exposure to 1-5 mM glutamate. Although the biochemical events have been well studied, little has been done to identify the transcriptional/translational changes which contribute to the glutamate-induced pathway of programmed cell death. Changes in gene expression clearly play a role in the cell death cascade since macromolecular synthesis is required early in the death pathway.

Using an experimental nerve cell model for oxidative stress and an expression cloning strategy, a gene involved in oxidative stress-induced programmed cell death was identified which both mediates the cell death program and regulates GSH levels. Two stress-resistant clones were isolated which contain antisense gene fragments of the translation initiation factor eIF2 $\alpha$  and express a low amount of eIF2 $\alpha$ . Sensitivity is restored when the clones are transfected with full length eIF2 $\alpha$ ; transfection of wild-type cells with the truncated eIF2 $\alpha$  gene confers resistance. The phosphorylation of eIF2 $\alpha$  also results in resistance to oxidative stress. In wild-type cells oxidative stress results in rapid glutathione depletion, a large increase in peroxide levels, and an influx of Ca<sup>2+</sup>. In contrast, the resistant clones maintain high glutathione levels and show no elevation in peroxides or Ca<sup>2+</sup> when stressed, and the glutathione synthetic enzyme  $\gamma$ -glutamyl cysteine synthetase (GCS) is elevated. The change in GCS is regulated by a translational mechanism. eIF2 $\alpha$  is therefore a critical regulatory factor in the response of nerve cells to oxidative stress and in the control of the major intracellular antioxidant, GSH, and may play a central role in the many neurodegenerative diseases and toxicities associated with oxidative stress. The manuscript describing this work is in the appendix.

### C. *Oxidative glutamate toxicity and excitotoxicity*

The physiological consequences of extracellular glutamate are mediated by three classes of membrane proteins within the central nervous system (CNS). These are ionotropic glutamate receptors, metabotropic glutamate receptors, and the cystine/glutamate antiporter. Ionotropic glutamate receptors have two known roles. They are responsible for the majority of excitatory neurotransmission within the CNS and also for a great deal of CNS pathology. In cases of stroke or trauma, excessive extracellular glutamate leads to nerve cell death via the activation of NMDA receptors. This phenomenon, which can be reproduced in cell culture is termed excitotoxicity. In contrast to ionotropic glutamate receptors, the metabotropic glutamate receptors (mGluRs) are G-protein coupled membrane proteins with a wide variety of biological functions. As described above, a third target for extracellular glutamate in the CNS is the inhibition of the glutamate/cystine antiporter which results in a form of oxidative stress and cell death called oxidative glutamate toxicity. The glutamate/cystine antiporter couples the import of cystine to the export of glutamate.

Concentrations of extracellular glutamate as low as 100 $\mu$ M, which is well below the level of extracellular glutamate found in models of stroke and trauma, completely inhibit the uptake of cystine. Cystine is required for the synthesis of the potent intracellular reducing agent glutathione (GSH). When GSH is depleted by extracellular glutamate, cells die from a form of programmed cell death.

The potential role of oxidative glutamate toxicity in ischemia and trauma is not understood, but there have been strong indications that several cell death pathways are involved in the excitotoxicity cascade. In localized cerebral infarction, the neurons in the epicenter die rapidly, while those more distal remain viable for several hours. Multiple forms of nerve cell death have also been identified in excitotoxic CNS primary culture paradigms following exposure to glutamate. In primary cultures of cerebellar granule cells exposed to glutamate, there is a rapid necrotic phase, followed by delayed apoptotic-like cell death. During oxygen-glucose deprivation of primary mouse cortical cultures or organotypic cultures of the rat hippocampus, some cell death occurs from non-ionotropic receptor-mediated mechanisms. All of these observations are consistent with in vivo data which show that non-receptor mediated programmed cell death may occur following ischemic insults. In addition, a number of parameters change dramatically during CNS stress which lead to the observed high exogenous glutamate. These include the direct release of glutamate from cells, the enzymatic conversion of glutamine to glutamate, and the shut down of nerve and glial glutamate uptake systems by pro-oxidant conditions. It is therefore of interest to determine if oxidative glutamate toxicity can play a significant role in nerve cell death associated with the excitotoxicity cascade.

In the manuscript attached to the appendix, we show that a portion of the cell death associated with NMDA receptor initiated excitotoxicity can be caused by oxidative glutamate toxicity. In primary mouse cortical neurons cell death resulting from the short term application of 10 $\mu$ M glutamate can be divided into NMDA and non-NMDA receptor dependent phases. The non-NMDA receptor dependent component is associated with high extracellular glutamate and is inhibited by a variety of reagents which uniquely block oxidative glutamate toxicity. These include metabotropic glutamate receptor agonists, antioxidants, and a caspase inhibitors. In addition, it is shown that the concentration of extracellular glutamate rises to several hundred micromolar, probably due to the conversion of glutamine to glutamate in the culture medium (glutamine is equally high in CNS tissue) by the enzyme glutaminase released from lysed cells. These results suggest that oxidative glutamate toxicity toward neurons lacking functional NMDA receptors can be a component of the excitotoxicity initiated cell death pathway.

### **KEY RESEARCH ACCOMPLISHMENTS**

- The Bax gene product is not involved in oxidative glutamate toxicity or excitotoxicity.
- The translation initiation factor eIF2 $\alpha$  can serve as a switch which determines whether nerve cells live or die during oxidative stress. The eIF2 $\alpha$  functions by regulating the level of intracellular glutathione by determining the level of the glutathione synthetic enzyme, GCS.
- The unique programmed cell death pathway, oxidative glutamate toxicity, is a component of the widely studied but little understood excitotoxicity cascade, which is involved in many forms of oxidative stress induced by trauma and disease.

### **REPORTABLE OUTCOMES**

Three manuscripts (appended).

A Ph.D. student (Shirlee Tan) who worked on eIF2 $\alpha$  graduated.

Research experience for a pre-med student (Dana Piasecki) working on the project.

### **CONCLUSIONS**

The above data led to a much better understanding of the mechanisms involved in the nerve cell death pathway initiated by neurotoxins such as glutamic acid. This information will help us design drugs and treatments which block neurotoxicity. It is the goal of next year's work to use the knowledge we have gained to identify compounds which block the oxidative death pathway and to identify additional molecular components of the pathway. To do this we will use DNA chips to

look for changes in gene expression rather than differential display as suggested in the original proposal. The chip technology has recently become functional at The Salk Institute, and it is much more efficient and less prone to artifact than differential display.

## **REFERENCES**

Appended manuscripts (see below).

## **APPENDIX**

- Dargusch, R., D. Piasecki, S. Tan, Y. Liu, and D. Schubert (2000). The role of Bax in glutamate induced nerve cell death. *J. Neurochem.*, in press.
- Schubert, D., and D. Piasecki (2000). Oxidative glutamate toxicity can be a component of the excitotoxicity cascade. *J. Neurosci.*, submitted.
- Tan, S., N. Somia, P. Maher, and D. Schubert (2000). Regulation of neuronal antioxidant metabolism by translation initiation factor-2 alpha. *Neuron*, submitted.

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## **The Role of Bax in Glutamate Induced Nerve Cell Death**

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**Abstract:**

The role of the Bax gene product was examined in three forms of cortical nerve cell death in primary cultures. These include spontaneous cell death, oxidative glutamate toxicity in which exogenous glutamate inhibits cystine uptake resulting in toxic oxidative stress, and ionotropic glutamate receptor-mediated excitotoxicity following a brief exposure to 10 $\mu$ M glutamate. Primary cortical and hippocampal neuron cultures were established from embryos of Bax  $-/+$  x Bax  $-/+$  matings and the embryos genotyped and assayed for cell death in the three experimental paradigms. Cell death induced by oxidative glutamate toxicity and glutamate-mediated excitotoxicity was not altered in the Bax  $-/-$  homozygous knock-out animals. In contrast, there was about a 50% inhibition of spontaneous cell death. These results suggest that a classical Bax-dependent apoptotic pathway contributes to the spontaneous cell death which takes place when nerve cells are initially exposed to cell culture conditions. A Bax-dependent programmed cell death pathway is not, however, utilized in oxidative glutamate toxicity and NMDA receptor-mediated excitotoxicity following a brief exposure to low concentrations of glutamate.

**Keywords:** Bax, knock-out, oxidative glutamate toxicity

**Running Title:** *Bax and nerve cell death*

## INTRODUCTION

The Bcl-2 family of proteins has been implicated as a necessary intermediate in the death of a wide variety of cell types caused by a large number of different agents (for recent review, see Chao and Korsmeyer, 1998). Within this family, some members inhibit cell death (such as Bcl-2 and Bcl-x<sub>L</sub>), while others promote cell death (for example Bax and Bad). A variety of approaches have suggested that the Bcl-2 family regulates at least some forms of cell death in the nervous system. For example, Bax knockout mice have increased cell numbers within the nervous system relative to control animals, as do animals which overexpress Bcl-2 (Farlie et al., 1995; Korsmeyer, 1999). In contrast, there is excessive cell death in Bcl-2 knockout mice and a promotion of apoptosis in mice which overexpress Bax (Knudson and Korsmeyer, 1997). In nerve cell culture paradigms, there have been a large number of studies which implicate the Bax/Bcl-2 pathway in nerve cell death. The best studied of these is the withdrawal of trophic support from neurotrophin dependent cell cultures (see for example, Deckwerth et al., 1996). Other examples include ionizing radiation-induced apoptosis (Chong et al., 2000) and p53 induced apoptosis (Bernard et al., 1998; Xiang et al., 1998; Cregan et al., 1999). In the vast majority of these experimental systems it has been concluded that the Bax pathway is required for the completion of the cell death program.

There are, however, two forms of nerve cell death which have been studied less extensively than trophic factor withdrawal with respect to the potential involvement of the Bax/Bcl-2 pathway. These are a form of oxidative stress induced cell death caused by glutamate, called oxidative glutamate toxicity (for review, see Maher and Schubert, 2000), and excitotoxicity caused by a brief exposure to low concentrations of glutamate. Ionotropic glutamate receptors have two known roles. They are responsible for both the majority of the excitatory neurotransmission within the CNS (for review, see Gasic and Hollmann, 1992), and also for a great deal of CNS pathology. In cases of stroke or trauma, excessive extracellular glutamate leads to nerve cell death via the activation of NMDA receptors (Rothman and Olney, 1986).

This phenomenon, which can be reproduced in cell culture (Choi, 1987; Rothman, 1985), is termed excitotoxicity (Olney, 1986). Excitotoxicity is thought to be mediated primarily by the entry of calcium ions through NMDA receptors (Choi, 1987; Connor et al., 1987; Garthwaite and Garthwaite, 1986; Hyrc et al., 1997).

Another prevalent form of nerve cell death is that caused by oxidative stress associated with many pathologies (for review, see Coyle and Puttfarcken, 1993). Perhaps the best model system for examining oxidative stress in the CNS is oxidative glutamate toxicity. Initially described by Murphy et al., in 1989, oxidative glutamate toxicity occurs when nerve cells are exposed to high exogenous concentrations of glutamate (Murphy et al., 1989). Extracellular glutamate inhibits the  $x_c^-$  cystine/glutamate antiporter, a protein complex which is responsible for the import of cystine in exchange for the export of glutamate (Sato et al., 1999). In the absence of cystine, glutathione (GSH), the major cellular antioxidant, is depleted, leading to oxidative stress and ultimately cell death. Although a number of requirements for cell death via this pathway have been identified, the potential role of the Bax/Bcl-2 family is unknown. In this manuscript we ask if Bax is required for cell death caused by excitotoxicity and oxidative stress, as well as the spontaneous cell death seen following the introduction of nerve cells in culture.

## MATERIALS and METHODS

### Cell culture

Primary cultures of cortical neurons which reproducibly die by excitotoxicity were prepared by combining aspects of two published protocols (Dugan et al., 1995; Rose et al., 1993). E14 Balb/c mouse embryo cortices were minced and treated with 0.1% trypsin for 20 min. Following centrifugation, the cells were resuspended in B27 Neurobasal medium (GIBCO, Boston, MA) plus 10% fetal calf serum and dissociated by repeated pipetting through a 1ml blue Eppendorf pipette tip. The cells were then plated in 96 well polylysine and laminin coated microtiter plates in B27 Neurobasal plus 10% fetal calf serum and 20% glial growth conditioned medium



prepared according to Dugan and colleagues (Dugan et al., 1995). The growth conditioned medium improved plating efficiency by about 30%. Two days later the medium was aspirated and replaced by serum-free B27-Neurobasal medium plus 10 $\mu$ g/ $\mu$ l cytosine arabinoside. The cultures were used without media change between 7 and 12 days after plating and were essentially free of astrocytes (Brewer et al., 1993).

For glutamate toxicity assays, the culture medium was moved with a multichannel pipetter to a new 96 well plate and the cells exposed to 10  $\mu$ M glutamate in a HEPES buffered salt solution (HCSS, Rose et al., 1993), containing 120mM NaCl, 5.4 mM KCl; 0.8 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 15 mM glucose and 20mM HEPES, pH 7.4. In some cases, 1 $\mu$ M glycine was included, but this had no net effect on excitotoxic death. After 10 min at room temperature, the HCSS was aspirated and the original growth medium returned to the cells. In all experiments test and control cells were exposed to identical conditioned media.

### MTT assay

Cell survival was determined by the MTT [3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as described in (Liu et al., 1997), which correlates with cell death as determined by trypan blue exclusion and a colony-forming assay (Davis and Maher, 1994). Twenty hours after the addition of glutamate, 10  $\mu$ l of the MTT solution (2.5 mg/ml) is added and the cells are incubated for 3 hrs at 37°C. 100  $\mu$ l of the solubilization solution (50% dimethylformamide and 20% SDS, pH 4.8) is added to the wells, and the next day the absorption values at 570 nm are measured. The results are expressed relative to the controls specified in each experiment and are expressed as the mean of triplicate determinations plus or minus the standard error of the mean.



### Western blotting

For Western blotting, cortical cells were collected directly into Laemmli buffer (Laemmli, 1970). Cell lysates (30µg per lane) were resolved in 12% polyacrylamide gels containing SDS and electrophoretically transferred to hybridization membranes (Micron Separations Inc., Westboro, MA). The membrane was first probed with a rabbit anti-Bax antiserum (N-20, Santa Cruz Biotechnology, Santa Cruz, CA) at 1µg/ml and then with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody at a dilution of 1:20,000. The antibody conjugates were detected using a chemiluminescence Western blot kit (Amersham, Buckinghamshire, England).

### Bax knockout mice

Heterozygous Bax mice were obtained from Dr. Stanley Korsmeyer (Deckwerth et al., 1996) and bred both to maintain the colony and as a source of embryos which lack Bax protein. Genomic DNA was extracted from tissue samples (0.5 cm lengths of tail from weanlings, 10-20 mg of brain from embryos) using the DNeasy Tissue Kit (Qiagen Inc., Valencia CA) following the suggested protocol. Relevant segments of DNA were amplified using AmpliTaq DNA Polymerase (Perkin Elmer, Boston MA) in accordance with the protocol provided by S. Korsmeyer and developed by M. Knudson. The following primers were used: BaxIN5R (Bax Intron 5 reverse primer) 5'-GTT GAC CAG AGT GGC GTA GG-3'; NeoR, (Neo/PGK reverse primer) 5'-CCG CTT CCA TTG CTC AGC GG-3' (Since Neo is in a reverse orientation it amplifies in the forward direction with Bax primer IN5R); BaxEX5F (Bax Exon 5 forward primer) 5'-GAG CTG ATC AGA ACC ATC ATG-3'. Reaction conditions were: 5min. 94°C, cycle: 1 min. 94°C, 1 min. 62°C, 1.5 min. 72°C. The cycle was repeated 30 times followed by a 7 min. elongation step at 72°C. Reactions were in 25µl volumes using all three primers. PCR products were resolved with 1.5x agarose gels.

## RESULTS

While the role of Bax has been studied extensively in several experimental paradigms for apoptosis, there has been no work on its role in nerve cell death caused by oxidative glutamate toxicity and only one manuscript which examined the role of Bax and P53 in nerve cell death caused by continuous exposure to relatively high concentrations of glutamate/kainate (Xiang et al., 1998). There is, however, little published material examining the role of Bax in excitotoxicity. We therefore examined the potential role of Bax in nerve cell death by asking how cells isolated from the nervous system of Bax knockout mice responded to oxidative stress, NMDA receptor-mediated excitotoxicity and spontaneous cell death in culture. If Bax is involved in the cell death mechanism, then it would be expected that cells lacking this protein would die less efficiently than wild type cells. As controls we used staurosporine induced apoptosis, processes known to be regulated by the Bax/Bcl-2 pathways (Rodriguez et al., 1996). The following paragraphs present the results of experiments designed to test the role of Bax in nerve cell death.

### **Bax expression is not required for excitotoxic and oxidative stress induced nerve cell death**

To determine if Bax expression is required for the forms of cell death which occur in NMDA receptor-mediated excitotoxicity as well as oxidative glutamate toxicity (a model for oxidative stress, (Murphy et al., 1989; Tan et al., 1998a; 1998b), E14 cortical neurons were cultured either for 2 days (oxidative glutamate toxicity) or 10 days (excitotoxicity) and assayed for sensitivity to glutamate. Cells from individual embryos were used and their genotype determined by PCR analysis. Figure 1 shows PCR patterns and Bax protein expression in cortical cells from a few embryos, confirming the absence of Bax protein expression in the homozygous Bax deleted animals. Bax is present in the heterozygous animals as expected.

The excitotoxic assay, based upon the activation of NMDA ionotropic glutamate receptors in 10 day old cortical cultures (Schubert and Piasecki, 2000) showed that in wild type,

heterozygous, and homozygous Bax knockout cells, there is no difference in cell death caused by low concentrations of glutamate (Figs. 2,3). The excitotoxic response is completely blocked by the NMDA receptor antagonist AP-5, showing that the cell death cascade is at least initiated by NMDA receptor activation.

In contrast to older cultures, mouse cortical neurons cultured for only 2 days express very low levels of any class of ionotropic glutamate receptors and therefore do not respond to excitotoxic insults (Murphy et al., 1989; Schubert and Piasecki, 2000). Although these cells lack ionotropic receptors, higher concentrations of glutamate block cystine uptake, causing a depletion of glutathione and a form of programmed cell death (Murphy et al., 1989; Tan et al., 1998a; 1998b). When cultures from Bax  $-/-$  or Bax  $+/+$  embryos are continuously exposed to concentrations of glutamate between 0.1 and 5mM for 24 hours there is half maximal cell death at 1mM glutamate in both sets of cultures. It can, therefore, be concluded that the expression of Bax is not required for nerve cell death observed in oxidative stress or NMDA receptor-mediated excitotoxicity.

### **Bax expression is required for staurosporine induced death**

As a positive control to establish that the cortical neurons were in fact functioning properly, an additional cell death pathway was examined in which a role for Bax has been established. Staurosporine and two day old cultures of cortical neurons were used. Staurosporine, a potent phosphatase inhibitor, causes Bax-dependent apoptosis (Ackermann et al., 1999). Figure 3 shows that the genomic deletion of Bax promoted cell survival from staurosporine induced cell death. Therefore it is clear that within the same population of cells both Bax-dependent and Bax-independent forms of programmed cell death can occur.

### **Bax expression is required for some of the initial spontaneous cell death in cortical neuron cultures**

With the culture conditions frequently used for primary cultures of CNS tissue, there is usually a rapid die-off of neurons during the first 2 days in culture, resulting in the loss of over 50% of the cells (Yankner et al., 1990). If this process is due to a classic apoptotic mechanism, possibly caused by growth factor withdrawal, then it would be expected that this loss would be significantly reduced in cells from homozygous Bax-deleted mice relative to their wild type littermates. Figure 4 shows that there are about twice the number of surviving cortical neurons in culture after 4 days in cultures from Bax  $-/-$  knockout mice than in the Bax  $+/+$  controls.

### **DISCUSSION**

The following conclusions may be drawn from the above data. 1. The deletion of the Bax gene partially rescues cortical neurons from the spontaneous cell death which occurs once dissociated cells are placed in cell culture. 2. Bax gene expression is not required for the death of cortical neurons caused by NMDA glutamate receptor-mediated excitotoxicity or glutamate-induced oxidative stress (oxidative glutamate toxicity). 3. The deletion of Bax does, however, protect cortical neurons from staurosporine induced cell death, suggesting that Bax-dependent apoptosis is responsible for the majority of the cell death in this system.

When CNS neurons are dissociated and placed in cell culture, there is a rapid die-off of cells until the cultures reach a stable cell number (see for example, Yankner et al., 1990). The cell depletion is probably due to the loss of neurotropic support, suggesting that death occurs by an apoptotic mechanism analogous to that caused by the withdrawal of NGF from cultured sympathetic neurons. Since Bax is required for cell death to occur in NGF-deprived sympathetic neurons (Deckwerth et al., 1998) as well as hyperpolarized cerebellar granule cells (Miller et al., 1997), it is likely that at least some of the spontaneous cell death in cortical primary cultures should be rescued by the deletion of Bax. Figure 4 shows that there is indeed a 2.5-fold increase

in cell number after 4 days in culture for Bax  $-/-$  cortical neurons relative to wild type Bax  $+/+$  cultures.

The activation of the NMDA class of glutamate receptors causes rapid cell death by a process which has primarily been defined as necrosis, although some data have suggested that apoptotic mechanisms may also be employed (Ankacrona et al., 1995). The influx of  $\text{Ca}^{++}$  through open NMDA channels is thought to initiate the excitotoxic cascade, but the overproduction of reactive oxygen species (ROS) and mitochondrial dysfunction are also involved (Dugan et al., 1995; Patel et al., 1996; Schinder et al., 1996). Figures 2 and 3 show that unlike spontaneous cell death in the primary cultures, excitotoxic cell death initiated by a brief 10 min exposure to  $10\mu\text{M}$  glutamate is not altered by the deletion of the Bax gene. Cell death in this system is exclusively mediated by NMDA receptors and is completely blocked by the NMDA specific antagonist AP-5 (Fig. 2B, (Schubert and Piasecki, 2000). It is therefore unlikely that nerve cell death via excitotoxicity involves the classical Bax-dependent apoptotic pathway. These data are in agreement with those of (Miller et al., 1997), who showed that the death of cerebellar granule cells initiated by NMDA (a form of excitotoxicity) is also unaffected in cells from Bax  $-/-$  mice relative to control Bax  $+/+$  mice.

The above data are, however, at odds with those of another experimental paradigm which concludes that Bax expression is required for excitotoxic cell death (Xiang et al., 1998). In the Xiang manuscript, cortical neurons were exposed to high ( $50\mu\text{M}$ ) concentrations of glutamate or kainate continuously for 3 days before cell viability was determined. In contrast, our data were obtained after a brief 10 min exposure to a low  $10\mu\text{M}$  glutamate, and cell death monitored 24 hrs later. It has been well established that both the mechanism and the type of cell death which occurs both *in vivo* and in cell culture depends upon both the concentration and duration of exposure to glutamate or glutamate agonist (for review, see Choi, 1992). Rapidly triggered excitotoxicity as used in this manuscript and slowly triggered excitotoxicity as described by Xiang and colleagues is probably mediated by different sets of ionotropic glutamate receptors

and results in physiologically distinct cell death pathways (Choi, 1992). It is therefore not unlikely that the Bax dependencies for cell death in the two experimental systems are different.

Oxidative glutamate toxicity is a form of glutamate-induced cell death which is independent of ionotropic glutamate receptors, but which shares some intermediate steps with excitotoxicity such as ROS production (Murphy et al., 1989). Most mammalian cells synthesize little or no cysteine, and are therefore dependent upon the import of extracellular cystine for both amino acids. The inhibition of the unique cystine/glutamate antiporter (Sato et al., 1999) by high concentrations of exogenous glutamate reduces intracellular cysteine and leads to severe oxidative stress and cell death. This pathway can, in fact, be a component of the excitotoxicity cascade when there is a high concentration of extracellular glutamate (Schubert and Piasecki, 2000). Oxidative glutamate toxicity has several features of classical apoptosis, including the requirement for macromolecular synthesis and for caspase activation (Tan et al., 1998a). However, the morphological criteria for classical apoptosis are not seen, nor is there DNA breakdown (Tan et al., 1998a; 1998b). Despite the overlap of the oxidative glutamate toxicity pathway with some aspects of classical apoptosis, there is no alteration in the kinetics or glutamate concentration dependence of cell death in cortical nerve cells derived from Bax  $-/-$  or Bax  $-/+$  mice relative to Bax  $+/+$  animals (Figs. 2,3). It is therefore unlikely that Bax is involved in cell death caused by oxidative glutamate toxicity. It has, however, been shown that the overexpression of Bcl-2 can protect PC12 cells from oxidative glutamate toxicity (Behl et al., 1993). This is not surprising since Bax and Bcl-2 can function independently to regulate cell death (Knudson and Korsmeyer, 1997). Bcl-2 can act as a pro-oxidant and can protect cells from oxidative stress when overexpressed by upregulating antioxidant defense mechanisms, therefore making cells more resistant to additional insults (Steinman, 1995).

In summary, the above data derived from primary cortical cultures of Bax  $-/-$  mice show that the Bax gene product is not required for nerve cell death pathways initiated by oxidative glutamate toxicity or by excitotoxicity initiated via the short term activation of NMDA receptors

by low concentrations of glutamate. They are in agreement with the exceptionally heterogeneous literature on cell death within the nervous system which shows that nerve cells can die by a variety of cell death pathways. The initiation of each pathway is, however, strictly dependent upon the nature of the insult.

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## REFERENCES

- Ackermann E. J., Taylor J. K., Narayana R. and Bennett C. F. (1999) The role of antiapoptotic Bcl-2 family members in endothelial apoptosis elucidated with antisense oligonucleotides. *J. Biol. Chem.* **274**, 11245-11252.
- Ankacrona M., Dypbukt J. M., Bonfoco E., Zhivotovsky B., Orrenius S., Lipton S. A. and Nicotera P. (1995) Glutamate-induced neuronal death: A succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15**, 961-973.
- Behl C., Hovey L., Krajewski S., Schubert D. and Reed J. C. (1993) BCL-2 prevents killing of neuronal cells by glutamate but not by amyloid beta protein. *Biochem. Biophys. Res. Comm.* **197**, 949-956.
- Bernard R., Dieni S., Rees S. and Bernard O. (1998) Physiological and induced neuronal death are not affected in NSE-bax transgenic mice. *J. Neurosci. Res.* **52**, 247-259.
- Brewer G. J., Torricelli J. R., Evege E. K. and Price P. J. (1993) Optimized survival of hippocampal neurons in B27-supplemented neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35**, 567-576.
- Chao D. T. and Korsmeyer S. J. (1998) BCL-2 family: regulators of cell death. *Annu. Rev. Immunol.* **16**, 395-419.
- Choi D. W. (1987) Ionic dependence of glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* **7**, 369-379.
- Choi D. W. (1992) Excitotoxic cell death. *J. Neurobiol.* **23**, 1261-1275.



- Chong M. J., Murray M. R., Gosink E. C., Russell H. R., Srinivasan A., Kapsetaki M., Korsmeyer S. J. and McKinnon P. J. (2000) Atm and Bax cooperate in ionizing radiation-induced apoptosis in the central nervous system. *Proc. Natl. Acad. Sci. USA* **97**, 889-894.
- Connor J. A., Tseng H. Y. and Hockberger P. E. (1987) Depolarization- and transmitter-induced changes in intracellular  $Ca^{2+}$  of rat cerebellar granule cells in explant cultures. *J. Neurosci.* **7**, 1384-1400.
- Coyle J. T. and Puttfarcken P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262**, 689-95.
- Cregan S. P., MacLaurin J. G., Craig C. G., Robertson G. S., Nicholson D. W., Park D. S. and Slack R. S. (1999) Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. *J. Neurosci.* **19**, 7860-7869.
- Davis J. B. and Maher P. (1994) Protein kinase C activation inhibits glutamate-induced cytotoxicity in a neuronal cell line. *Brain Res.* **652**, 169-173.
- Deckwerth T. L., Easton R. M., Knudson C. M., Korsmeyer S. J. and Johnson E. M., Jr. (1998) Placement of the BCL2 family member BAX in the death pathway of sympathetic neurons activated by trophic factor deprivation. *Exp. Neurol.* **152**, 150-162.
- Deckwerth T. L., Elliott J. L., Knudson C. M., Johnson E. M., Jr., Snider W. D. and Korsmeyer S. J. (1996) BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron* **17**, 401-411.

- Dugan L. L., Bruno V. M. G., Amagasu S. M. and Giffard R. G. (1995) Glia modulate the response of murine cortical neurons to excitotoxicity: Glia exacerbate AMPA neurotoxicity. *J. Neurosci.* **15**, 4545-4555.
- Dugan L. L., Sensi S. L., Canzoniero L. M. T., Handran S. D., Rothman S. M., Lin T.-S., Goldberg M. P. and Choi D. W. (1995) Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J. Neurosci.* **15**, 6377-6388.
- Farlie P. G., Dringen R., Rees S. M., Kannourakis G. and Bernard O. (1995) Bcl-2 transgene expression can protect neurons against developmental and induced cell death. *Proc. Natl. Acad. Sci. USA* **92**, 4397-4401.
- Garthwaite G. and Garthwaite J. (1986) Neurotoxicity of excitatory amino acid receptor agonists in rat cerebellar slices: dependence on calcium concentration. *Neurosci. Lett.* **66**, 193-198.
- Gasic G. P. and Hollmann M. (1992) Molecular neurobiology of glutamate receptors. *Annu. Rev. Physiol.* **54**, 507-536.
- Hyrk K., Handran S. D., Rothman S. M. and Goldberg M. P. (1997) Ionized intracellular calcium concentration predicts excitotoxic neuronal death: Observations with low-affinity fluorescent calcium indicators. *J. Neurosci.* **17**, 6669-6677.
- Knudson C. M. and Korsmeyer S. J. (1997) Bcl-2 and Bax function independently to regulate cell death. *Nat. Genet.* **16**, 358-363.
- Korsmeyer S. J. (1999) BCL-2 gene family and the regulation of programmed cell death. *Cancer Res.* **59**, 1693s-1700s.

- Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Liu Y., Peterson D. A., Kimura H. and Schubert D. (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J. Neurochem.* **69**, 581-93.
- Maher P. and Schubert D. (2000) Signaling by reactive oxygen species in the nervous system. *Cell. Molec. Biol.* **57**, in press.
- Miller T. M., Moulder K. L., Knudson C. M., Creedon D. J., Desmukh M., Korsmeyer S. J. and Johnson E. M., Jr. (1997) Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. *J. Cell Biol.* **139**, 205-217.
- Murphy T. H., Miyamoto M., Sastre A., Schnaar R. L. and Coyle J. T. (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* **2**, 1547-1558.
- Murphy T. H., Miyamoto M., Sastre A., Schnaar R. L. and Coyle J. T. (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* **2**, 1547-1558.
- Olney J. W. (1986) Inciting excitotoxic cytocide among central neurons, in *Advances in Experimental Medicine and Biology* (Schwarcz R., and Ben-Ari Y., eds), pp. 632-645. Plenum Press, New York.

- Patel M., Day B. J., Crapo J. D., Fridovich I. and McNamara J. O. (1996) Requirement for superoxide in excitotoxic cell death. *Neuron* **16**, 345-355.
- Rodriguez I., Matsuura K., Khatib K., Reed J. C., Nagata S. and Vassalli P. (1996) A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection. *J. Exp. Med.* **183**, 1031-1036.
- Rose K., Goldberg M. P. and Choi D. W. (1993) Cytotoxicity in murine neocortical cell culture. *Meth. Toxicol.* **1A**, 46-60.
- Rothman S. M. (1985) The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J. Neurosci.* **5**, 1483-1489.
- Rothman S. M. and Olney J. W. (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann. Neurol.* **19**, 105-111.
- Sato H., Tamba M., Ishii T. and Bannai S. (1999) Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J. Biol. Chem.* **274**, 11455-11458.
- Schinder A. F., Olson E. C., Spitzer N. C. and Montal M. (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J. Neurosci.* **16**, 6125-6133.
- Schubert D. and Piasecki D. (2000) Oxidative glutamate toxicity can be a part of the excitotoxicity cascade. *Eur. J. Neurosci.* *submitted*.
- Tan S., Sagara Y., Liu Y., Maher P. and Schubert D. (1998a) The regulation of reactive oxygen species production during programmed cell death. *J. Cell Biol.* **141**, 1423-1432.

- Tan S., Wood M. and Maher P. (1998b) Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. *J. Neurochem.* **71**, 95-105.
- Xiang H., Kinoshita Y., Knudson C. M., Korsmeyer S. J., Schwartzkroin P. A. and Morrison R. S. (1998) Bax involvement in p53-mediated neuronal cell death. *J. Neurosci.* **18**, 1363-1373.
- Yankner B. A., Duffy L. K. and Kirschner D. A. (1990) Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science* **250**, 279-282.

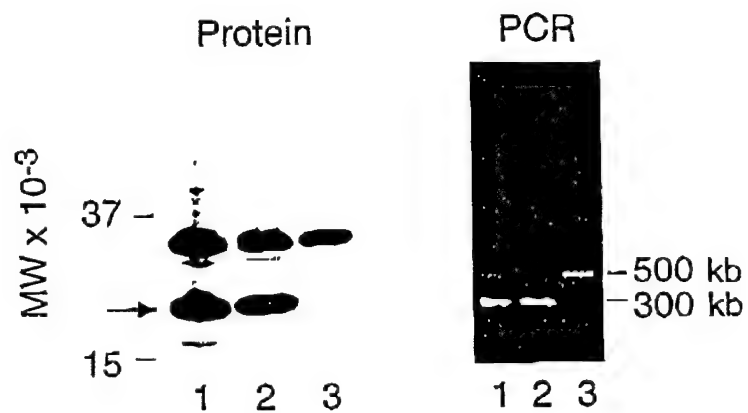
## FIGURE LEGENDS

**Fig. 1.** Bax is not expressed in homozygous Bax deleted animals. The genotype of individual embryos was determined as described in Materials and Methods using PCR primers. Cortical tissue from the same animals was separated on SDS acrylamide gels and immunoblotted with anti-Bax antiserum. Lane 1 Bax +/+; Lane 2 Bax -/+; Lane 3 Bax -/-. Arrow indicates position of Bax in the western blots. The bands above 25,000 MW are nonspecific. PCR detection of genotype. Lane 1 Bax -/+; Lane 2 Bax +/+; Lane 3 Bax -/-.

**Fig. 2.** Oxidative glutamate toxicity and excitotoxicity. Cells from individual E14 day embryos of Bax heterozygous matings were plated in 96 well microtiter dishes and assayed for either oxidative glutamate toxicity in 2 day cultures lacking ionotropic glutamate receptors (A) or excitotoxicity in 10 day old cultures expressing ionotropic glutamate receptors (B) as described in Materials and Methods. Pieces of brain tissue from each embryo was used to isolate DNA and to determine the Bax genotype of the individual animals. Results are presented as the percent of the control (non treated) cell viability at each concentration of glutamate. o - o, Bax -/-; x - x, Bax +/+;  $\Delta$  -  $\Delta$ , Bax -/+.  $\bullet$  -  $\bullet$ , 100 $\mu$ M AP-5 Bax -/-;  $\nabla$  -  $\nabla$ , 100  $\mu$ M AP-5 Bax +/+. The results are presented as the mean plus or minus the standard error of the mean, n=6.

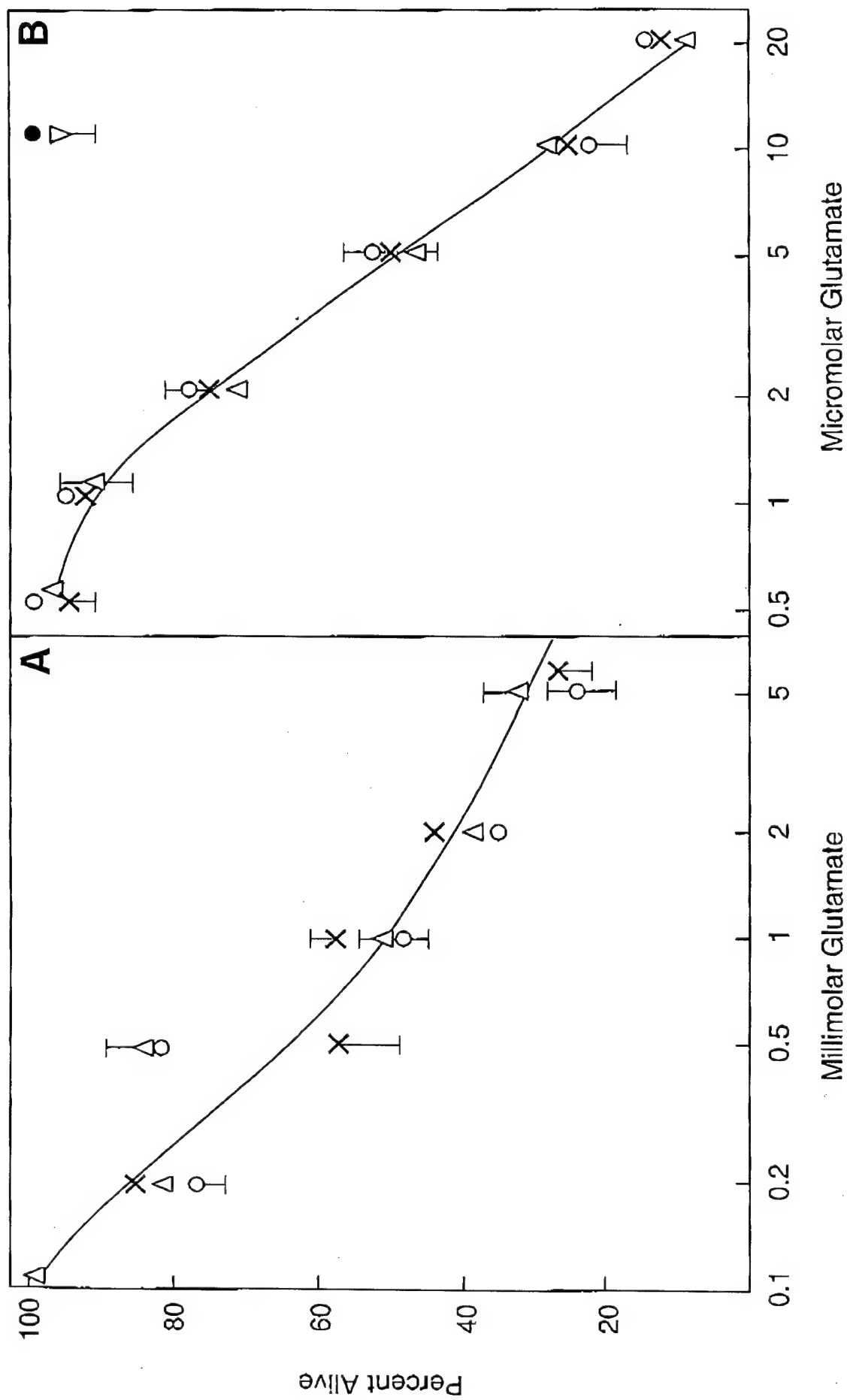
**Fig. 3.** Cell death in Bax knockout mice. Cortical nerve cells from individual E14 embryos resulting from a Bax -/+ x Bax -/+ mating were assayed for excitotoxicity, oxidative glutamate toxicity, or staurosporine induced cell death as described in Materials and Methods. The results are presented as the mean plus or minus the standard error of the mean for at least 5 embryos. \*, statistically different from control ( $p < .01$ ). The data are calculated as percent of change in cell viability relative to the control cultures derived from wild type mice (Bax +/+) with the following conditions staurosporine (STP, 200nM); excitotoxicity (Excito, 5 $\mu$ M glutamate); Oxidative glutamate toxicity (OxGlu, 2mM glutamate).

**Fig. 4.** Time course of cell loss in cortical primary cultures. E14 cortical neurons were plated on polylysine at  $5 \times 10^5$  cells per 35mm tissue culture dish and viable cells determined daily as described in Materials and Methods. The relative cell number is plotted against days in culture. x - x, Bax  $-/-$ ; ● - ●, Bax  $+/+$ .

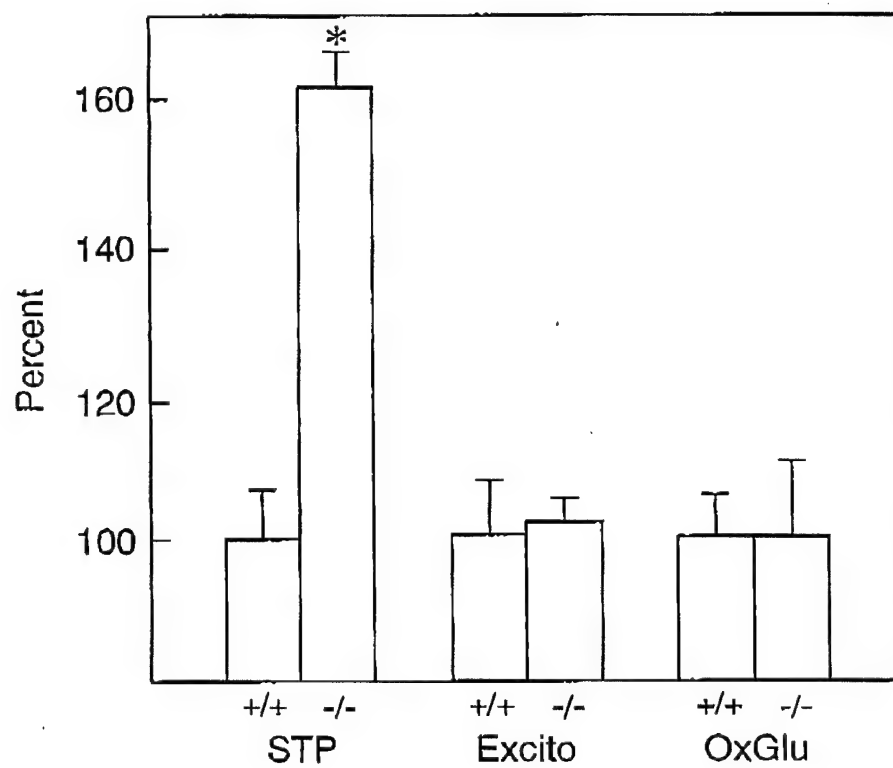


Dargusch et al.  
Figure 1

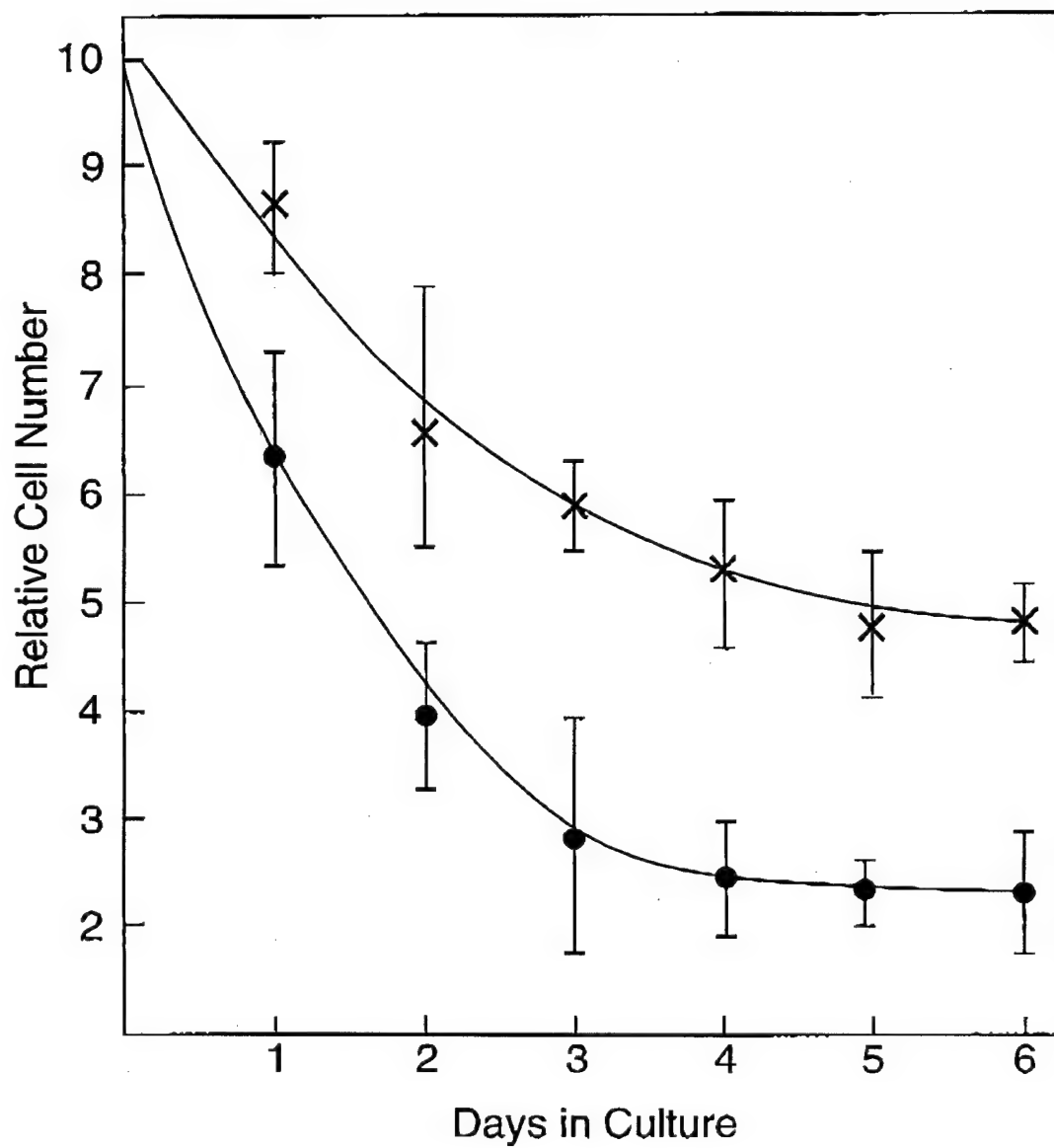




Dargusch et al.  
Figure 2



Dargusch et al.  
Figure 3



DSFIG192.GRAPH

Figure 4

# **Oxidative Glutamate Toxicity can be a Component of the Excitotoxicity Cascade**

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**Abstract**

Along with ionotropic and metabotropic glutamate receptors, the cystine/glutamate antiporter,  $x_c^-$ , may play a critical role in CNS pathology. High levels of extracellular glutamate inhibit the import of cystine, resulting in the depletion of glutathione and a form of cell injury called oxidative glutamate toxicity. Here we show that a portion of the cell death associated with NMDA receptor initiated excitotoxicity can be caused by oxidative glutamate toxicity. In primary mouse cortical neurons cell death resulting from the short term application of 10 $\mu$ M glutamate can be divided into NMDA and non-NMDA receptor dependent phases. The non-NMDA receptor dependent component is associated with high extracellular glutamate and is inhibited by a variety of reagents which block oxidative glutamate toxicity. These results suggest that oxidative glutamate toxicity toward neurons lacking functional NMDA receptors can be a component of the excitotoxicity initiated cell death pathway.

## Introduction

The physiological consequences of extracellular glutamate are mediated by three classes of membrane proteins within the central nervous system (CNS). These are ionotropic glutamate receptors, metabotropic glutamate receptors, and the cystine/glutamate antiporter. Ionotropic glutamate receptors have two known roles. They are responsible for the majority of excitatory neurotransmission within the CNS and also for a great deal of CNS pathology. In cases of stroke or trauma, excessive extracellular glutamate leads to nerve cell death via the activation of NMDA receptors (Rothman and Olney, 1986). This phenomenon, which can be reproduced in cell culture (Choi, 1987; Rothman, 1985) is termed excitotoxicity (Olney, 1986). In contrast to ionotropic glutamate receptors, the metabotropic glutamate receptors (mGluRs) are G-protein coupled membrane proteins with a wide variety of biological functions (Nakanishi, 1994). Finally, a third target for extracellular glutamate in the CNS is the inhibition of the glutamate/cystine antiporter  $x_c^-$  which results in a form of oxidative stress and cell death called oxidative glutamate toxicity (Murphy *et al.*, 1989). The glutamate/cystine antiporter couples the import of cystine to the export of glutamate (Sato *et al.*, 1999). Concentrations of extracellular glutamate as low as 100  $\mu$ M, which is well below the level of extracellular glutamate found in models of stroke and trauma (see for example, McAdoo *et al.*, 1999), completely inhibit the uptake of cystine (Sagara and Schubert, 1998). Cystine is required for the synthesis of the potent intracellular reducing agent glutathione (GSH). When GSH is depleted by extracellular glutamate, cells die from a form of programmed cell death (Tan *et al.*, 1998a).

The potential role of oxidative glutamate toxicity in ischemia and trauma is not understood, but there have been strong indications that several cell death pathways are involved in the excitotoxicity cascade. In localized cerebral infarction, the neurons in the epicenter die rapidly, while those more distal remain viable for several hours (Siesjo, 1992). Multiple forms of nerve cell death have also been identified in excitotoxic CNS primary culture paradigms following exposure to glutamate (for review, see Choi, 1992). In primary cultures of cerebellar granule cells exposed to glutamate, there is a rapid necrotic phase, followed by delayed apoptotic-like cell death

(Ankacrona *et al.*, 1995). During oxygen-glucose deprivation of primary mouse cortical cultures or organotypic cultures of the rat hippocampus, some cell death occurs from non-ionotropic receptor-mediated mechanisms (Gwag *et al.*, 1995; Newell *et al.*, 1995). All of these observations are consistent with in vivo data which show that non-receptor mediated programmed cell death may occur following ischemic insults (Linnik *et al.*, 1993; MacManus *et al.*, 1993; Okamoto *et al.*, 1993; Shigeno *et al.*, 1990). In addition, a number of parameters change dramatically during CNS stress which lead to the observed high exogenous glutamate. These include the direct release of glutamate from cells, the enzymatic conversion of glutamine to glutamate, and the shut down of nerve and glial glutamate uptake systems by pro-oxidant conditions (see Discussion). It is therefore of interest to determine if oxidative glutamate toxicity can play a significant role in nerve cell death associated with the excitotoxicity cascade.

## Materials and Methods

### Cell Culture

Primary cultures of cortical neurons which reproducibly die by excitotoxicity were prepared by combining aspects of two published protocols (Dugan *et al.*, 1995; Rose *et al.*, 1993). E14 Balb/c mouse embryo cortices were minced and treated with 0.1% trypsin for 20 min. Following centrifugation, the cells were resuspended in B27 Neurobasal medium (GIBCO, Grand Island, NY) plus 10% fetal calf serum and dissociated by repeated pipetting through a 1ml blue Eppendorf pipette tip. The cells were then plated in 96 well polylysine and laminin coated microtiter plates (Bectin-Dickerson) in B27 Neurobasal plus 10% fetal calf serum and 20% glial growth conditioned medium prepared according to Dugan and colleagues (Dugan *et al.*, 1995). The growth conditioned medium improved plating efficiency by about 30%. Two days later the medium was aspirated and replaced by serum-free B27-Neurobasal medium plus 10 $\mu$ g/ $\mu$ l cytosine arabinoside. The cultures were used without media change between 7 and 12 days after plating and were essentially free of astrocytes (Brewer *et al.*, 1993).

For glutamate toxicity assays, test drugs (e.g. antioxidants) were added 30 min prior to glutamate exposure. For glutamate toxicity, the culture medium was moved with a multichannel pipetter to a new 96 well plate and the cells exposed to glutamate (usually 10 $\mu$ M) in a HEPES buffered salt solution (HCSS, (Rose *et al.*, 1993)), containing 120mM NaCl, 5.4 mM KCl; 0.8 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 15 mM glucose and 20mM HEPES, pH 7.4. In some cases, 1 $\mu$ M glycine was included, but this had no net effect on excitotoxic death. After 10 min at room temperature, the HCSS was aspirated and the original growth medium returned to the cells. In some cases the NMDA antagonist AP5 was added at this point to inhibit the down stream activation of glutamate receptors.

#### *MTT Assay*

Cell survival was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as described (Schubert *et al.*, 1992), which correlates with cell death as determined by trypan blue exclusion and a colony-forming assay (Davis and Maher, 1994). Twenty hours after the addition of glutamate, 10 $\mu$ l of the MTT solution (2.5 mg/ml) is added to each well and the cells are incubated for 3 hrs at 37°C. 100  $\mu$ l of solubilization solution (50% dimethylformamide and 20% SDS, pH 4.8) is then added to the wells, and the next day the absorption values at 570 nm are measured. The results are expressed relative to the controls specified in each experiment and are expressed as the mean of triplet determinations plus and minus the standard error of the mean.

#### *Western Blotting*

For Western blotting, cells were collected directly in Laemmli buffer (Laemmli, 1970). Cell lysates were resolved in 10% polyacrylamide gels containing SDS and electrophoretically transferred to hybridization membranes (Micron Separations Inc., Westboro, MA). The membrane was first probed with a rabbit antiserum at a dilution of 1:2000 and then with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody at a dilution of 1:20,000. The antibody conjugates were detected using a chemiluminescence Western blot kit (Amersham,



Buckinghamshire, England). Glutamate assays in growth conditioned medium were done by both mass spectroscopy and standard amino acid analysis with similar results (Iwabuchi *et al.*, 1994). The mass spectroscopy data are presented.

**Reagents.** The mGluR agonists and antagonists were all from Tocris Cookston, and mGluR 1 and 2/3 antisera and anti-NMDA antisera were from Chemicon (Temecula, CA). Anti-mGluR5 was a gift from Dr. R. Gereau (The Salk Institute, La Jolla, CA). The remaining reagents were from Sigma.

## Results

### *Cortical Neuron Cell Death can be Initiated by a Purely NMDA Receptor Dependent Mechanism*

As outlined above, there is some evidence for a non-ionotropic glutamate receptor component of the excitotoxicity cascade, but there have been only limited attempts to isolate and study this event. To do so, a number of criteria should be met. These include reproducibility, a pure nerve cell population to avoid confounding interactions with glia, a quantitative cytotoxic assay, and a system where the process is initiated by the activation of a single class of ionotropic receptors, ideally NMDA receptors. By combining and modifying a number of published procedures (see, for example, Dugan *et al.*, 1995; Rose *et al.*, 1993), a cell culture system was devised which meets these criteria. Briefly, E14 mouse cortical neurons are dissociated and plated into 96 well microtiter plates in Neurobasal medium containing B27 supplements (Brewer *et al.*, 1993) and fetal calf serum. Two days later the medium is replaced with serum-free B27 supplemented medium alone containing cytosine arabinoside. The experiments are done between 7 and 14 days after plating, and cell viability is usually determined by the reduction of MTT (Liu *et al.*, 1997) 24 hrs after a 10 min exposure to glutamate. After 8 days in culture, the cells are killed by glutamate with an EC<sub>50</sub> of approximately 2  $\mu$ M and by NMDA with an EC<sub>50</sub> of 20  $\mu$ M. AMPA and kainate are not toxic to these cells unless concentrations in excess of 100  $\mu$ M are used (Fig. 1). The toxicity of 10  $\mu$ M glutamate is completely blocked by the NMDA receptor antagonists AP5, DCQX, and MK-801, but not by the kainate/AMPA antagonists CNQX, GYKI-52466 or AMOA (Table 1). These

data show that the cytotoxic cascade in this culture system is initiated exclusively by the activation of NMDA receptors, therefore meeting the criterium for excitotoxicity as initially defined by Olney (Olney, 1986).

### *Cell Death is Rapidly Initiated*

To determine how rapidly cells die under the experimental conditions outlined above, cultures were exposed to 10 $\mu$ M glutamate for 10 min, followed by a 3 hr MTT viability assay at various times after glutamate exposure. The results were confirmed by visual assays, including propidium iodide exclusion. Figure 2A shows that most of the cell death is quite rapid, with maximal levels around 4 hr post-glutamate exposure. The duration of exposure to 10 $\mu$ M glutamate required to elicit maximum cell death is also short. When cells are exposed to 10 $\mu$ M glutamate for various lengths of time, followed by a viability assay 24 hr later, cell death was significant after 1 min and maximum with a 3 to 4 min exposure (Fig. 2B). All cell death was prevented by the inclusion of 100 $\mu$ M AP5 in the glutamate incubation medium. Therefore there is a very efficient coupling between NMDA receptor activation and the initiation of the cell death pathways.

### *Cell Death can be Divided into Three Components*

While the initiation of cell death is totally dependent upon the activation of NMDA receptors, it is possible that other forms of cell death are hidden within the ionotropic receptor initiated process. To isolate a possible non-NMDA receptor component, cells were exposed to 10 $\mu$ M glutamate for 10 min and then cultured continuously in the presence or absence of AP5, a potent NMDA antagonist which completely blocks glutamate toxicity in these cultures (Fig. 1, Table 1). Figure 3 shows that at 8 days in culture, two components of the excitotoxicity cascade are revealed by this procedure. Approximately 80% of the cells are killed by a 10 min exposure to 10 $\mu$ M glutamate, and none are killed when AP5 is present with glutamate. However, if AP5 is added immediately after the exposure to glutamate, about 30% of the cells are rescued from cell death (arrow A). It follows that the 30% of the cells which are rescued by AP5 require NMDA receptor activation after

glutamate exposure, while the remaining 50% (arrow B) must be killed by either the initial exposure to glutamate through the activation of NMDA receptors, or by a downstream mechanism which is independent of the NMDA receptor. If AP5 is present during the exposure to glutamate and then removed from the cultures, there is still no cell death, for under these conditions glutamate cannot activate receptors and initiate the cascade. These observations are consistent with previous observations which show that a significant fraction of cells destined to die following glutamate exposure can be rescued by NMDA antagonists applied after the initial glutamate exposure (Hartley and Choi, 1989; Manev *et al.*, 1989; Rothman *et al.*, 1987).

The interpretation of these data, and the basis for the following experiments, is that the activation of NMDA receptors during the 10 min exposure to 10 $\mu$ M glutamate initiates the death of a population of cells which is represented within the "B" component. This event triggers two additional responses due to the release of intracellular glutamate from dying cells. One is the subsequent activation of NMDA receptors on additional cells, resulting in more receptor dependent cell death (population A), and the other outcome is the death of a population of cells which do not have functional NMDA receptors (a subset of population B). The experiments below will define this latter population.

#### *Glutamate Receptor Expression Changes with Length of Time in Culture*

It has been repeatedly observed that the efficiency of excitotoxic cell death is dependent upon the length of time cells have been maintained in culture (see, for example, Dugan *et al.*, 1995). This is presumably due to the time required for the cells to express functional ionotropic receptors. To assay the distribution of NMDA receptor versus non-NMDA receptor mediated killing as a function of time in culture, the experiment described in Fig. 3 was repeated on days 7 through 11 of cell culture. The fraction of the total nerve cell culture which is killed by a 10 min exposure to 10 $\mu$ M glutamate increases from 40% at day 7 to about 80% on days 10 and 11 (Fig. 4). In contrast, about 60% of the cells which die are rescued by the post-glutamate addition of AP5 at day 7. This decreases to 20% between days 10 and 11.

The observation that the total number of cells killed increases with culture age suggests either that the level of NMDA receptor expression increases or its coupling to relevant second message systems is dependent upon the amount of time the neurons are in culture. Since one NMDA receptor subunit, NR1, is common to most NMDA ionotropic channels (for review, see Akazawa *et al.*, 1994), the expression of this subunit was followed by western blotting as a function of time in culture. Figure 5A, part IV shows that the expression of the NR1 receptor dramatically increases between days 3 and 10 in culture, suggesting that NMDA receptor availability may be limiting in the NMDA receptor mediated killing. Concomitant with culture age is an increase in neurite density (data not presented). Actin is a major component of neurites, and the amount of actin in neuronal cultures correlates with neurite density. Figure 5A, part V shows that there is an increase in actin accumulation which closely parallels that of NR1, suggesting that most of the NR1 may be associated with neurites.

In addition to ionotropic receptors, glutamate activates metabotropic receptors (mGluRs). mGluR activation has been associated with a variety of physiological processes, including protection from oxidative glutamate toxicity (Sagara and Schubert, 1998). Therefore the expression of mGluRs 1, 3 and 4, and 5 were monitored by western blotting in the same lysates as NR1 and actin. Figure 5 shows that all of these receptors are expressed in the cortical cultures, but that their expression patterns vary. The expression of mGluRs 1 and 5 increases with time in culture until day 7, after which their expression declines. In contrast, the expression of mGluRs 2 and/or 3 increase with culture age in a manner similar to NR1 and actin.

#### *Oxidative Glutamate Toxicity is a Component of Excitotoxicity*

Oxidative glutamate toxicity is a well studied programmed cell death pathway which is independent of ionotropic glutamate receptors (Murphy *et al.*, 1989; Li *et al.*, 1997a; 1997b; Maher and Davis, 1996; Tan *et al.*, 1998a; 1998b). If oxidative glutamate toxicity is a component of excitotoxicity then it should be inhibited by reagents which selectively block oxidative glutamate toxicity but not by AP5. If a compound blocks the NMDA mediated component in addition to

oxidative glutamate toxicity, then the whole cascade would be inhibited since its initiation is dependent upon NMDA receptor activation. Therefore, to determine if oxidative glutamate toxicity is involved in the excitotoxicity pathway, a variety of components which inhibit oxidative glutamate toxicity were screened in the excitotoxicity paradigm for their ability to block the B fraction of the excitotoxicity cascade (Fig. 3).

A defining characteristic of oxidative glutamate toxicity is that it is strongly inhibited by many antioxidants, including vitamin E (Murphy *et al.*, 1989). To determine if the B component shares this trait with oxidative glutamate toxicity, 8 day old cultures of cortical cells were pre-incubated for 30 min with 100 $\mu$ M  $\alpha$  tocopherol, followed by glutamate exposure and a 24 hr incubation with  $\alpha$  tocopherol plus or minus AP5. Figure 6A shows that part of the B phase of cell death is blocked by  $\alpha$  tocopherol, while the viability of the cells exposed to glutamate in the absence of AP5 is also increased by the same amount. This increase in viability is expected in the absence of AP5 since this condition contains both the non-NMDA receptor and NMDA receptor-mediated components of glutamate toxicity. Since half of the cells survive at day 8 in the presence of  $\alpha$  tocopherol and  $\alpha$  tocopherol has no effect on excitotoxicity at days 10 and 11 (data not presented),  $\alpha$  tocopherol must not block the NMDA receptor mediated excitotoxicity component. Although these results are consistent with oxidative glutamate toxicity being a component of the excitotoxicity cascade, a number of other reagents known to inhibit oxidative glutamate toxicity were also examined. These include the group I metabotropic glutamate receptor (mGluR1) agonists and a caspase inhibitor.

The activation of group I mGluRs protects cells from oxidative glutamate toxicity via the activation of the inositol triphosphate pathway (Sagara and Schubert, 1998). Using the same logic applied to the experiments with vitamin E, if oxidative glutamate toxicity is a component of excitotoxicity, then mGluRI agonists should inhibit part of component (B) of the cascade. Figures 6B and C show that two mGluR agonists, (R,S)-3,5-dihydroxyphenylglycine (DHPG) and trans-1-amino-1,3 cyclopentanedicarboxylic acid (ACPD), both protect from excitotoxic initiated glutamate damage in 8 day cultures. It has also been shown elsewhere that ACPD has a partial protective effect on NMDA-mediated excitotoxicity (Koh *et al.*, 1991). Another agent which

protects cortical neurons from oxidative glutamate toxicity is Ac-YVAD-cmk, a potent caspase inhibitor (Tan *et al.*, 1998a; 1998b). Figure 6D shows that this inhibitor protects cells in the presence of AP5 by about 20%. These data again substantiate the involvement of oxidative glutamate toxicity as the cause of between 20-30% of the cell death in the excitotoxicity cascade.

The vitamin E, the mGluR agonist, and the caspase inhibitor data show that under certain conditions excitotoxicity can be divided into 3 components, one of which has the characteristics of oxidative glutamate toxicity. In older cultures (10-11 days) only 20% of the cell death is blocked by the late application of AP5 and no cell death is blocked by the oxidative glutamate toxicity antagonists described above (Fig. 4 and data not shown). These data show that the oxidative glutamate toxicity component of excitotoxicity is transient in these cultures and strongly support the argument that vitamin E, DHPG, ACPD and YVAD do not inhibit the NMDA receptor mediated response. The transient nature of the response may be due to the fact that the NMDA receptor-mediated response is more efficient in older cultures due to higher receptor density or the loss of cells which do not express NMDA receptors from the older cultures. This would also result in a larger fraction of the cells being killed by initial glutamate exposure (a larger B component, Fig. 3).

#### *Soluble Glutamate Mediates Late Cell Death*

The above data show that there can be both an NMDA receptor and non-ionotropic receptor mediated component of excitotoxicity, and that the latter can be accounted for by the oxidative glutamate pathway. If this conclusion is valid then it should be possible to transfer the late toxicity via the growth conditioned medium and also to partially protect cells previously exposed to glutamate by replacing their conditioned medium with fresh medium. If positive, these experiments would also rule out the possibility that all of the "B" component cells are killed by the initial exposure to glutamate. Figure 7 shows that this is indeed the case. Conditioned medium replacement reduces subsequent cell death by about 30%, while about 30% of the cell death caused by the transfer of conditioned medium of cells treated for 10 min with 10 $\mu$ M glutamate to fresh

cells is not blocked by AP5 or AMPA/kainate antagonists (data not presented), but is blocked by the caspase inhibitor YVAD. Since these data are similar to those obtained by directly treating cells with AP5 and the caspase inhibitor, it is very likely that the late cell death observed in these cultures is due to free glutamate.

The amount of free glutamate in the culture medium was assayed as a function of time after the addition of 10 $\mu$ M glutamate for 10 minutes plus or minus 100 $\mu$ M AP5 to inhibit the excitotoxicity cascade. Figure 7 shows that the amount of free glutamate increased from undetectable levels (less than 10 $\mu$ M) to about 300  $\mu$ M over a period of 9 hrs. 300 $\mu$ M glutamate is sufficient to inhibit extracellular cystine uptake and deplete intracellular GSH in clonal nerve cells (Sagara and Schubert, 1998) and kill over 50% of the cells in this culture system via oxidative glutamate toxicity as determined by the long term exposure to glutamate in the presence of high concentrations of NMDA, AMPA, and kainate antagonists (Fig. 6A). These data clearly show that extracellular glutamate in these cultures can reach concentrations sufficient to cause damage via the oxidative glutamate toxicity pathway. The glutamate concentrations are higher than previously reported in cell culture systems (Strijbos *et al.*, 1996), most probably due to the absence of astrocytes to remove free glutamate.

## Discussion

The above data show that the excitotoxicity cascade can be experimentally divided into three discrete components, two requiring the activation of NMDA receptors. The initiation of the cell death pathway requires NMDA receptor activation, and a second NMDA receptor dependent phase takes place after a brief exposure to low concentrations of glutamate. In contrast, a distinct form of cell death can occur following glutamate exposure which is independent of ionotropic glutamate receptors. This pathway, which constitutes 20-30% of the total cell death in 8-9 day cultures, has characteristics of oxidative glutamate toxicity, for it is specifically inhibited by vitamin E, group I metabotropic receptor agonists, and a caspase inhibitor. These data explain earlier observations which show that there is significant cell death in excitatory amino acid toxicity, ischemia, and CNS

trauma which is independent of ionotropic glutamate receptors (see for example, Choi, 1992; Meldrum and Garthwaite, 1990).

In cultures of hippocampal neurons, about half of the cells can be rescued by applying NMDA antagonists following glutamate exposure (Hartley and Choi, 1989; Manev *et al.*, 1989; Rothman *et al.*, 1987). These data and those presented above show that there is an initial population of cells which is killed directly by glutamate exposure, and another population which dies later due to the activation of NMDA receptors. The late receptor mediated cell death could be due to either the requirement for a subset of NMDA receptors which respond to the higher concentrations of extracellular glutamate derived from cell lysis, or have a requirement for more prolonged exposure to cell derived glutamate. In our experiments, of the cells which cannot be rescued by the late application of AP5, about half die by a process with the characteristics of oxidative glutamate toxicity. The other half die due to the initial exposure to glutamate, and require NMDA receptor activation.

Previous studies have shown that the activation of different classes of ionotropic glutamate receptors is dependent upon both glutamate concentration, exposure time, and probably the cell population. For example, unlike for NMDA, a brief exposure of cortical cells to AMPA and kainate produces little cell death, but exposure of cells to these receptor agonists for hours produces extensive cell death (Choi *et al.*, 1989; Frandsen *et al.*, 1989). This may be because most AMPA/kainate receptors are relatively impermeable to  $\text{Ca}^{++}$ , requiring the activation of voltage dependent  $\text{Ca}^{++}$  channels for toxicity. In addition to exposure duration, AMPA/kainate receptor mediated cell death is much slower, requiring many hours for cell lysis to occur (Carriedo *et al.*, 1998; Choi, 1992), and these later forms of cell death have some characteristics of apoptosis (Choi and Rothman, 1990; Kure *et al.*, 1991; Portera-Cailliau *et al.*, 1997). However, since AMPA/kainate receptor antagonists have no effect in this culture system (Table 1), even when added after glutamate exposure (data not presented), it is unlikely that these receptors play a role in the cell death which occurs after transient glutamate exposure. However, consistent with most of the published literature is the observation that some downstream cell death occurs by a mechanism



which has many characteristics of programmed cell death, such as caspase activation (Tan *et al.*, 1998a; 1998b). This cell death pathway is oxidative glutamate toxicity.

Oxidative glutamate toxicity requires higher concentrations of glutamate than are necessary for NMDA receptor activation (Murphy *et al.*, 1989). Figure 7 shows that concentrations of extracellular glutamate in the 200-300 $\mu$ M range are present in cultured cells following initial excitotoxic cell lysis; these concentrations are sufficient to cause oxidative glutamate toxicity (Fig. 6A). Similar concentrations of extracellular glutamate have been reported in culture media of lysed neurons (Newcomb *et al.*, 1997) and in CNS trauma models, McAdoo *et al.*, 1999). Since the culture medium contains 2mM glutamine and nerve cells possess a very active enzyme, glutaminase, which converts glutamine to glutamate, initial nerve cell lysis releases this enzyme which, in the presence of abundant substrate, leads to an accumulation of glutamate in the culture medium (Newcomb *et al.*, 1997). The brain also contains concentrations of glutamine between 2 and 4 mM, with 0.5 mM found in cerebrospinal fluid (Matsumoto *et al.*, 1995). Since this culture system lacks glial cells and most of the nerve cells are rapidly damaged, there is no effective way of removing glutamate. During ischemia, trauma and other pro-oxidant conditions there is likely to be a loss of high affinity glutamate transporter function because these molecules are exquisitely sensitive to biological oxidants (for review, see Trotti *et al.*, 1998).

In oxidative glutamate toxicity, glutamate blocks the cystine/glutamate exchange system  $x_c^-$ , resulting in glutathione depletion and cell death (Murphy *et al.*, 1989). The molecular basis of  $x_c^-$  function has recently been described (Sato *et al.*, 1999). The exchange systems consist of two proteins, the heavy chain of 4F2 which is involved in several amino acid transport systems, and a 502 amino acid protein called XCT. Both XCT and 4F2hc are highly expressed in the brain (Kanai *et al.*, 1998; Sato *et al.*, 1999). Since the cells of the CNS contain sequestered concentrations of free glutamate in the millimolar range (Coyle *et al.*, 1981), it is probable that any cellular dysfunction, such as loss of energy metabolism or cell lysis, would create local concentrations of glutamate sufficient to inhibit glutamate uptake and subsequent glutathione synthesis in nearby cells. The EC<sub>50</sub> glutamate concentration for inhibiting cystine uptake is less than 100 $\mu$ M (Sagara

and Schubert, 1998), and about 200 $\mu$ M extracellular glutamate kills 50% of the cells via oxidative glutamate toxicity (Fig. 6A). This sequence of events could lead to cell injury or death in an autocatalytic manner, resulting in a gradient of injury radiating out from the site of the initial event, and there would be no requirement for ionotropic glutamate receptors for the cells to die. In addition, oxidative glutamate toxicity can generate even greater damage than excitotoxicity since neurons lacking ionotropic glutamate receptors are also killed. It is therefore of importance to understand the regulation of  $x_c^-$  in the brain as well as how oxidative glutamate toxicity kills neurons.

## References

- Akazawa C, Shigemoto R, Bessho Y, Nakanishi S, Mizuno N. (1994) Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. *J Comp Neurol* 347:150-160.
- Ankacrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P (1995) Glutamate-induced neuronal death: A succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* 15:961-973.
- Brewer GJ, Torricelli J R, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented neurobasal, a new serum-free medium combination. *J Neurosci Res* 35:567-576.
- Carriedo SG, Yin HZ, Sensi SL, Weiss JH (1998) Rapid  $\text{Ca}^{2+}$  entry through  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels triggers marked intracellular  $\text{Ca}^{2+}$  rises and consequent oxygen radical production. *J Neurosci* 18:7727-7738.
- Choi DW (1987) Ionic dependence of glutamate neurotoxicity in cortical cell culture. *J Neurosci* 7:369-379.
- Choi DW (1992) Excitotoxic cell death. *J. Neurobiol* 23:1261-1275.
- Choi DW, Rothman SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu Rev Neurosci* 13:171-182.
- Choi DW, Viseskul V, Amirthanayagam M, Monyer H (1989) Aspartate neurotoxicity on cultured cortical neurons. *J Neurosci Res* 23:116-121.
- Coyle JT, Bird SJ, Evans RH, Gulley RL, Nadler JV, Nicklas WJ, Olney JW (1981) Excitatory amino acid neurotoxins: selectivity and mechanisms of action. *Neurosci Res Prog Bull* 19:331-427.
- Davis JB, Maher P (1994) Protein kinase C activation inhibits glutamate-induced cytotoxicity in a neuronal cell line. *Brain Res* 652:169-173.

- Dugan LL, Bruno VMG, Amagasu SM, Giffard RG (1995) Glia modulate the response of murine cortical neurons to excitotoxicity: Glia exacerbate AMPA neurotoxicity. *J Neurosci* 15:4545-4555.
- Frandsen A, Drejer J, Schousboe A (1989) Direct evidence that excitotoxicity in cultured neurons is mediated via N-methyl-D-aspartate (NMDA) as well as non-NMDA receptors. *J Neurochem* 53:297-299.
- Gwag BJ, Lobner D, Koh JY, Wie MB, Choi DW (1995) Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen-glucose deprivation in vitro. *Neuroscience* 68:615-619.
- Hartley DM, Choi DW (1989) Delayed rescue of N-methyl-D-aspartate receptor-mediated neuronal injury in cortical culture. *J Pharmacol Exp Therap* 250:752-758.
- Iwabuchi , Kitazawa E, Kobayashi N., Watanabe H, Kanai M, Nakamura K (1994) Studies of drug metabolism using liquid chromatography/mass spectrometry: Comparison of three liquid chromatographic/mass spectrometric interfaces. *Biol Mass Spectrometry* 23:540-546.
- Kanai Y, Segawa H, Miyamoto K., Uchino H, Takeda, E, Endou H (1998) Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen. *J Biol Chem* 273:23629-23632.
- Koh J-Y, Palmer E, Cotman CW (1991) Activation of the metabotropic glutamate receptor attenuates N-methyl-D-aspartate neurotoxicity in cortical cultures. *Proc Natl.Acad Sci USA* 88:9431-9435.
- Kure S, Tominaga,T, Yoshimoto T., Tada K, Narisawa K (1991) Glutamate triggers internucleosomal DNA cleavage in neuronal cells. *Biochem Biophys Res Comm* 179:39-45.
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Li Y, Maher P, Schubert D (1997a) A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. *Neuron* 19:453-463.
- Li Y, Maher P, Schubert D (1997b) Requirement for cGMP in nerve cell death caused by glutathione depletion. *J Cell Biol* 139:1317-1324.

- Linnik MD, Zobrist RH, Hatfield MD (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 24:2002-2009.
- Liu Y, Peterson DA, Kimura H, Schubert D (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J Neurochem* 69:581-593.
- MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston E (1993) Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci Lett* 164:89-92.
- Maher P, Davis J (1996) The role of monoamine metabolism in oxidative glutamate toxicity. *J Neurosci* 16:6394-6401.
- Manev H, Favaron M, Guidotti A, Costa E (1989) Delayed increase of  $Ca^{2+}$  influx elicited by glutamate: Role in neuronal death. *Molec Pharmacol* 36:106-112.
- Matsumoto K, Lo E, Pierce AR, Halpern EF, Newcomb R (1995) *J Cerebr Blood Flow Metab* 16:114-124.
- McAdoo DJ, Xu G-Y, Robak G, Hughes MG (1999) Changes in amino acid concentrations over time and space around an impact injury and their diffusion through the rat spinal cord. *Exp Neurol* 159:538-544.
- Meldrum B, Garthwaite J (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. *TiPS* 11:379-387.
- Murphy TH, Miyamoto M, Sastre A, Schnaar RL, Coyle JT (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* 2:1547-1558.
- Nakanishi S. (1994) Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. *Neuron* 13:1031-1037.
- Newcomb R, Sun X, Taylor L, Curthoys N, Giffard RG (1997) Increased production of extracellular glutamate by the mitochondrial glutaminase following neuronal death. *J Biol Chem* 272:11276-11282.

- Newell D., Barth A, Papermaster V, Malouf AT (1995) Glutamate and non-glutamate receptor mediated toxicity caused by oxygen and glucose deprivation in organotypic hippocampal cultures. *J Neurosci* 15:7702-7711.
- Okamoto M, Matsumoto M, Ohtsuki T., Taguchi A, Mikoshica K, Yanagihara T, Kamada T (1993) Internucleosomal DNA cleavage involved in ischemia-induced neuronal death. *Biochem Biophys Res Commun* 196:1356-1362.
- Olney JW (1986) Inciting excitotoxic cytocide among central neurons. In: *Advances in Experimental Medicine and Biology* (Schwarcz R, Ben-Ari Y eds), pp. 632-645, New York:Plenum Press.
- Rose K, Goldberg MP, Choi DW (1993) Cytotoxicity in murine neocortical cell culture. *Meth Toxicol* 1A:46-60.
- Rothman SM (1985) The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J Neurosci* 5:1483-1489.
- Rothman SM, Olney JW (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann Neurol* 19:105-111.
- Rothman SM, Thurston JH, Hauhart RE (1987) Delayed neurotoxicity of excitatory amino acids in vitro. *Neuroscience* 22:471-480.
- Sagara Y, Schubert D (1998) The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. *J Neurosci* 18:6662-6671.
- Sato H, Tamba M, Ishii T, Bannai S (1999) Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J Biol Chem* 274:11455-11458.
- Schubert D, Kimura H, Maher P (1992) Growth factors and vitamin E modify neuronal glutamate toxicity. *Proc Natl Acad Sci (USA)* 89:8264-8267.
- Shigeno T, Yamasaki Y, Kato G, Kusaka K, Mama T, Takakura K, Graham DI, Furukawa S (1990) Reduction of delayed neuronal death by inhibition of protein synthesis. *Neurosci Lett* 120:117-119.

- Siesjo BK (1992) Pathophysiology and treatment of focal cerebral ischemia. *J Neurosurg* 77:169-184.
- Strijbos PJ, Leach MJ, Garthwaite J (1996) Vicious cycle involving Na<sup>+</sup> channels, glutamate release, and NMDA receptors mediates delayed neurodegeneration through nitric oxide formation. *J Neurosci* 16:5004-5013.
- Tan S, Sagara Y, Liu Y, Maher P, Schubert D (1998a) The regulation of peroxide production during programmed cell death. *J Cell Biol* 141:1423-1432.
- Tan S, Wood M, Maher P (1998b) Oxidative stress in nerve cells induces a form of cell death with characteristics of both apoptosis and necrosis. *J Neurochem* 71:95-105.
- Trotti D, Danbolt NC, Volterra A (1998) Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? *TIPS* 19:328-334.

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**Table 1**

Reagent	EC <sub>50</sub>		
	Day 8	Day 9	Day 10
GYKI-25466	>100μM	>100μM	>100μM
AMOA	>300μM	>300μM	>300μM
CNQX	>1000μM	>1000μM	>1000μM
MK-801	500nM	450nM	450nM
AP5	55μM	50μM	50μM
DCQX	100μM	100μM	100μM

Cells were exposed to varying concentrations of the reagents for 30 min before, during, and after exposure to 10μM glutamate for 10 min. Cell death was determined 24 hr later by the MTT assay, and the concentrations which protected 50% of the cells from 10μM glutamate toxicity are presented. The experiments were repeated at least 3 times with similar results.

> means that there is no effect at this concentration, the highest tested.

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### Figure Legends

**Figure 1.** *Ionotropic glutamate receptor mediated toxicity.* After 8 days in culture, E14 cortical neurons were exposed to the indicated reagents for 10 min and cell viability measured 24 hr later by the MTT assay as described in Methods. The results were confirmed by visual (trypan blue exclusion) assays. x – x, glutamate; O – O, NMDA;  $\Delta$  –  $\Delta$ , kainate;  $\nabla$  10 $\mu$ M glutamate plus 100  $\mu$ M AP5;  $\square$  –  $\square$ , AMPA.

**Figure 2.** *Temporal requirements for glutamate excitotoxicity.* A. Cells 9 days in culture were exposed to 10 $\mu$ M glutamate for 10 min, followed by a 3 hr MTT assay for viability at various times after glutamate exposure. For example, at 0 hr cells were exposed to glutamate and immediately assayed for viability in the 3 hr MTT assay; the 5 hr point is a 5 hr incubation after glutamate, followed by a 3 hr MTT assay. B. Cells were exposed to 10 $\mu$ M glutamate for 0 to 20 minutes, followed by the MTT viability assay 24 hr later. At the 30 sec time point, about 35% of the cells died during the next 24 hr. The results are the mean of triplicate determinations plus or minus the standard error of the mean.

**Figure 3.** *A portion of excitotoxic cell death is non NMDA receptor mediated.* After 8 days in culture, E14 cortical neurons were exposed for 10 min to the indicated concentrations of glutamate in the presence or absence of AP5 and then incubated for 24 hr in the presence or absence of AP5, at which time cell viability was monitored by the MTT assay. x – x, glutamate alone;  $\Delta$  –  $\Delta$ , glutamate + 100 $\mu$ M AP5 during and after the 10 min glutamate exposure; O – O, glutamate + 100 $\mu$ M AP5 added immediately after glutamate exposure. “A” indicates the fraction of cells which die after glutamate exposure by a NMDA receptor mediated process. “B” indicates the fraction of cells which die by virtue of the initial NMDA activation of the cell death pathway and those which die independently of the NMDA receptor after the initial exposure to glutamate.

**Figure 4.** *Changes in cell death mechanism as a function of time in culture.* E14 cortical cultures were monitored for glutamate induced cell death exactly as described in Fig. 3, but as a function of time in culture. The end-point that is plotted is plateau of killing by 10 $\mu$ M glutamate (Fig. 3).  $\Delta$  -  $\Delta$ , percent cells alive when 100 $\mu$ M AP5 was present during and after exposure to glutamate;  $\bullet$  -  $\bullet$ , percent of the initial cell population killed by glutamate (10 min exposure);  $\bigcirc$  -  $\bigcirc$ , percent of total cell death in the culture by indirect NMDA receptor activation (percent alive with AP5 present after glutamate exposure minus percent alive with glutamate alone, see Fig. 3"A");  $\square$  -  $\square$ , percent cells killed whose death is not blocked by the application of AP5 after glutamate. (100% minus the percent alive with AP5 added after glutamate exposure, see Fig. 3"B").  $\nabla$  -  $\nabla$ , the percent of cells which are rescued by 100 $\mu$ M DHPG (see Fig. 6B). The data are the mean plus or minus the standard error of the mean of 3 or 4 experiments.

**Figure 5.** *Expression of glutamate receptors as a function of time in culture.* Cell lysates were made from E14 cortical neurons cultured for 2 through 11 days. The lysates were then run on SDS acrylamide gels and immunoblotted with the indicated anti-receptor antibodies. Quantitation is via scanning the negatives. A. I. mGluR1; II. mGluR2/3; III. mGluR5; IV. NMDA NR1; V. actin. The experiments were repeated at least 3 times with similar results. B.  $\bullet$  -  $\bullet$ , mGluR1;  $\times$  -  $\times$ , mGluR2/3;  $\bigcirc$  -  $\bigcirc$ , mGluR5;  $\Delta$  -  $\Delta$ , NR1;  $\nabla$  -  $\nabla$ , actin, shown as percent of maximal expression.

**Figure 6.** *Conditions which block oxidative glutamate toxicity partially protect.* A. Alpha tocopherol protects from cell death. Cells cultured for 8 days were pretreated for 30 min with 100 $\mu$ M  $\alpha$  tocopherol (natural), exposed to 10 $\mu$ M glutamate for 10 min, and then returned to the original medium  $\pm$  AP5,  $\pm$   $\alpha$  tocopherol.  $\times$  -  $\times$ , glutamate alone;  $\bigcirc$  -  $\bigcirc$ , glutamate +  $\alpha$  tocopherol;  $\Delta$  -  $\Delta$ , glutamate + 100 $\mu$ M AP5 after glutamate exposure;  $\square$  -  $\square$ , glutamate +  $\alpha$  tocopherol + AP5 after glutamate.  $\nabla$  -  $\nabla$ , Cell viability after 24hrs following continuous exposure to glutamate plus 100 $\mu$ M AP5, 100 $\mu$ M GYKI-25466 and 500 $\mu$ M CNQX. B and C. Group I mGluR activation is protective. Cells were pretreated for 30 min with 100 $\mu$ M mGluR agonists DHPG (B) or ACPD

(C), followed by a 10 min exposure to the indicated concentrations of glutamate. The original culture medium was then returned to the cells along with the mGluR reagents and in some cases, 100 $\mu$ M AP5 to block down stream NMDA receptor activation. Cell viability was determined 24 hr later by the MTT assay. x - x, glutamate alone;  $\Delta$ , glutamate plus agonist; O - O, glutamate + AP5 after glutamate exposure;  $\square$  -  $\square$ , glutamate + AP5 after + agonist. **D.** A caspase inhibitor Ac-YVAD-cmk protects cells. Cells were exposed to 30 $\mu$ M Ac-YVAD-cmk for 30 min prior to exposure to 10 $\mu$ M glutamate. In some cases 100 $\mu$ M AP5 was present throughout. x - x, glutamate alone;  $\Delta$  -  $\Delta$ , glutamate plus caspase inhibitor; O - O, glutamate plus AP5;  $\square$  -  $\square$ , glutamate plus AP5 after plus caspase inhibitor.

**Figure 7.** *Toxicity is transferred by conditioned medium.* Cells were exposed to 10 $\mu$ M glutamate for 10 min, washed once, and returned to their original growth medium. At the indicated times, the cells were either given fresh culture medium ( $\Delta$  -  $\Delta$ ), the medium transferred to new cells of identical age in the presence (O - O) or absence (x - x) of 100 $\mu$ M AP5, or in the presence of 30 $\mu$ M Ac-YVAD-cmk + AP5 ( $\nabla$  -  $\nabla$ ). Cell viability was measured 24 hr later in all cultures. At 0,3,6 and 9 hours the amount of glutamate in the culture medium of cells exposed to 10 $\mu$ M glutamate for 10 min was determined in the presence ( $\square$  -  $\square$ ) or absence ( $\bullet$  -  $\bullet$ ) of 100 $\mu$ M AP5.

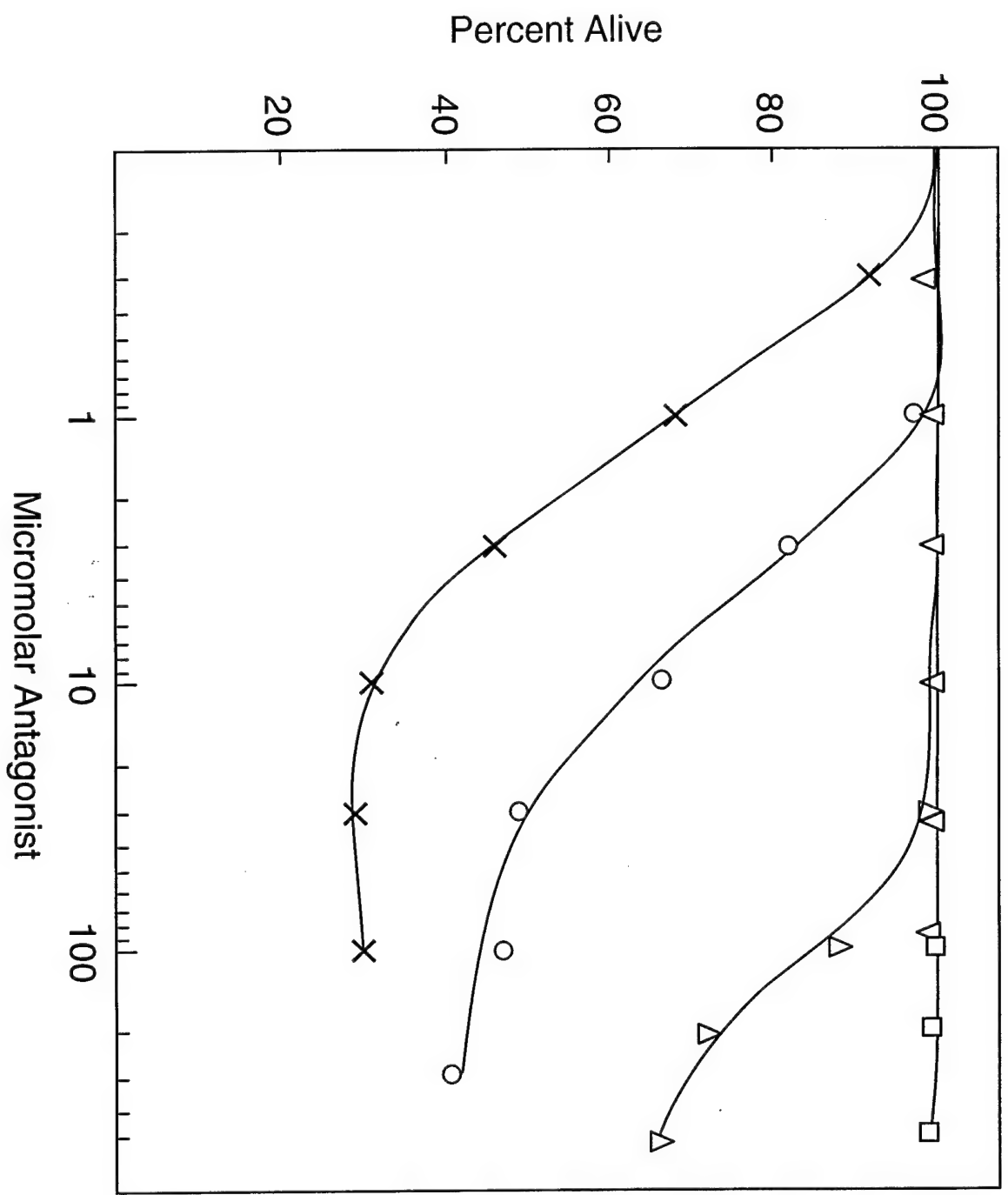


FIGURE 1

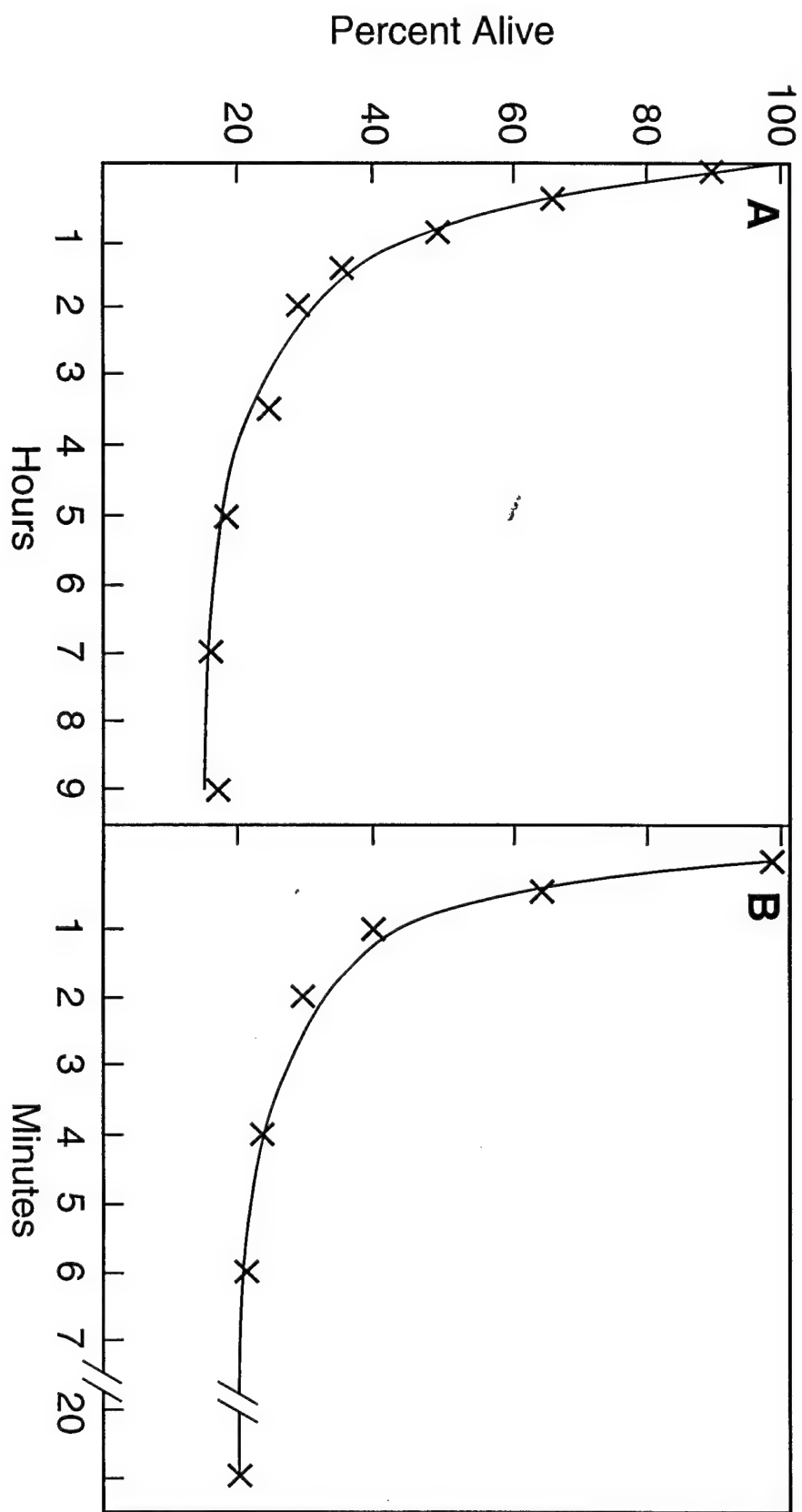


FIGURE 2

FIGURE 3

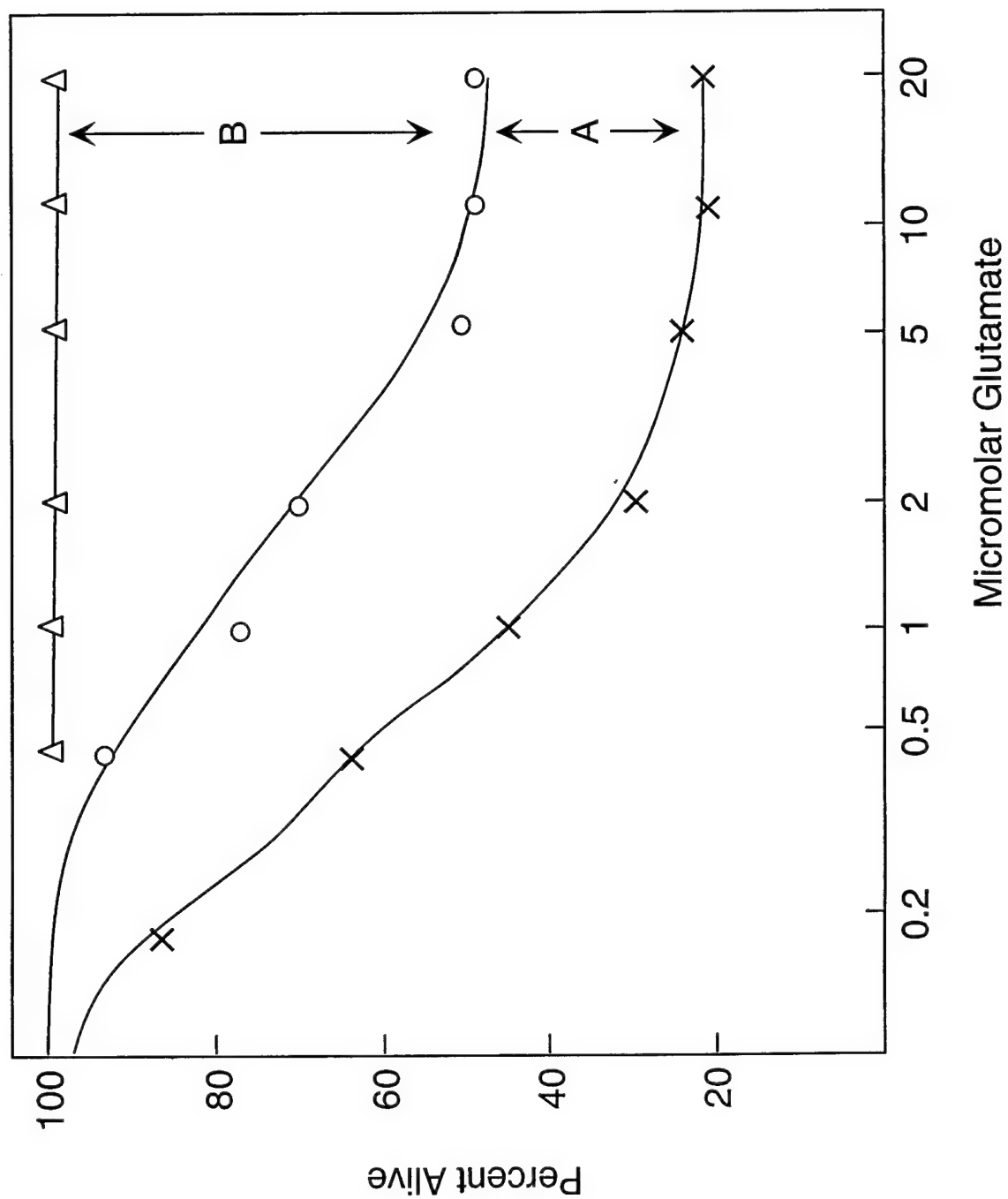
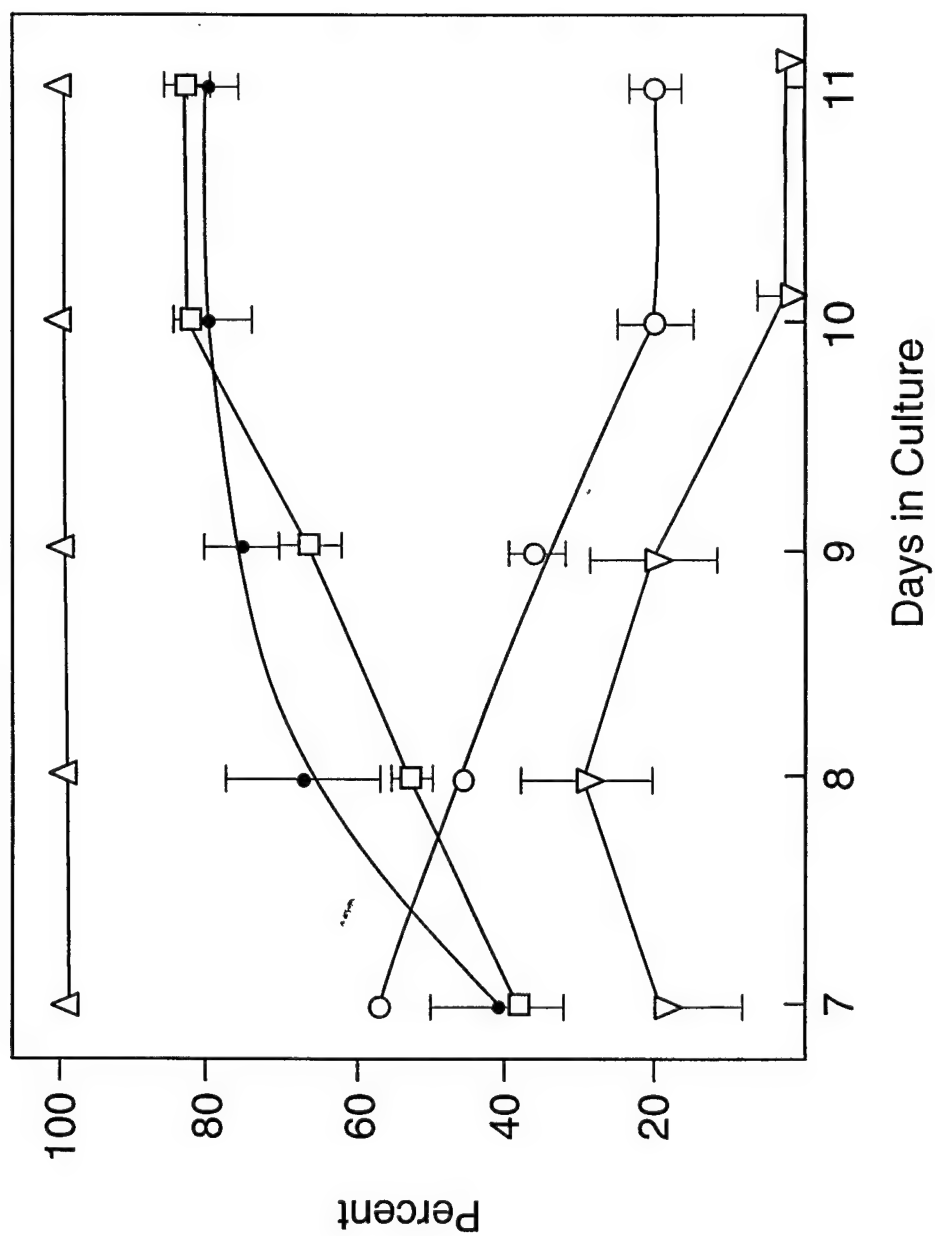


FIGURE 4



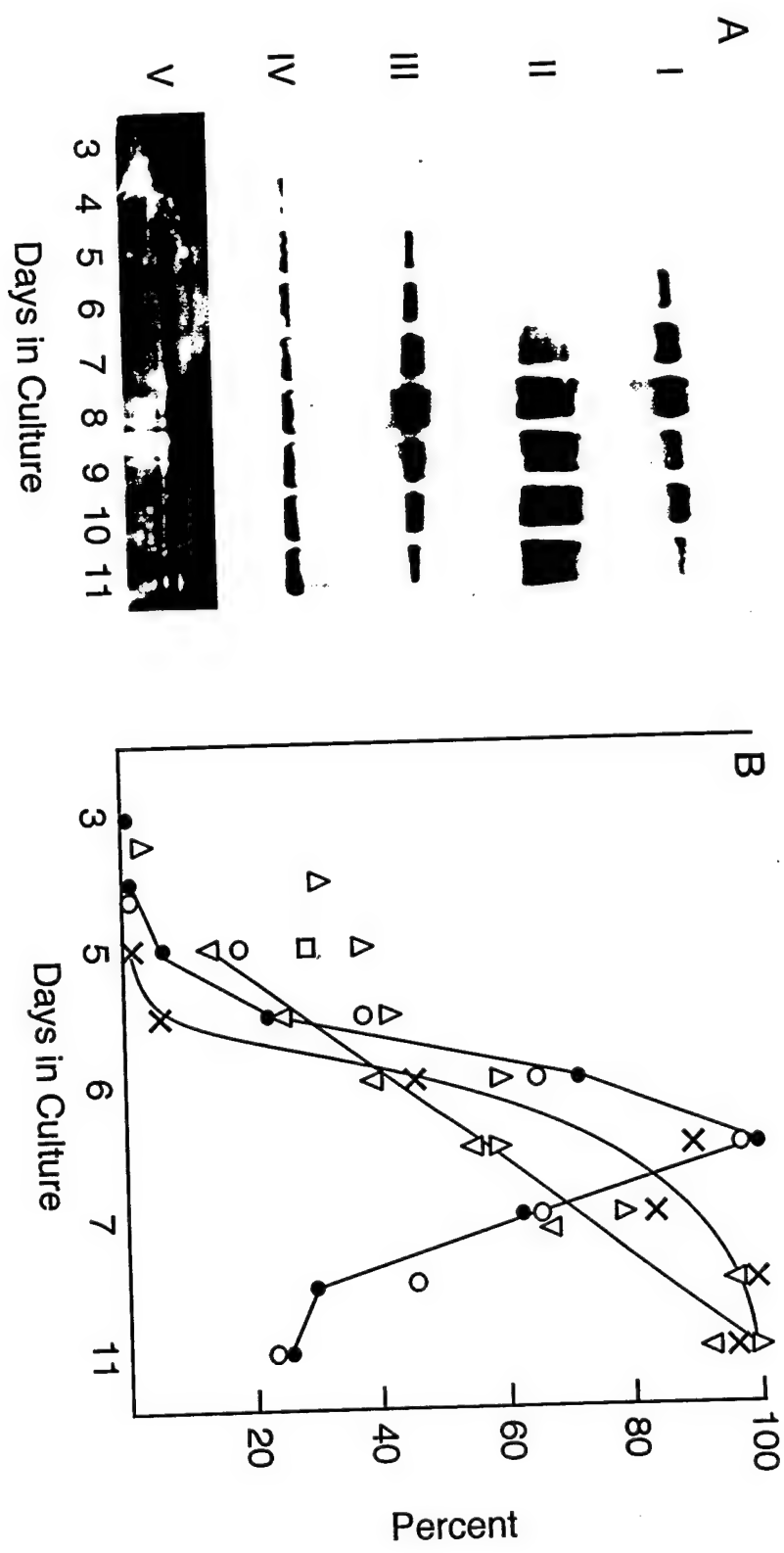


FIGURE 5



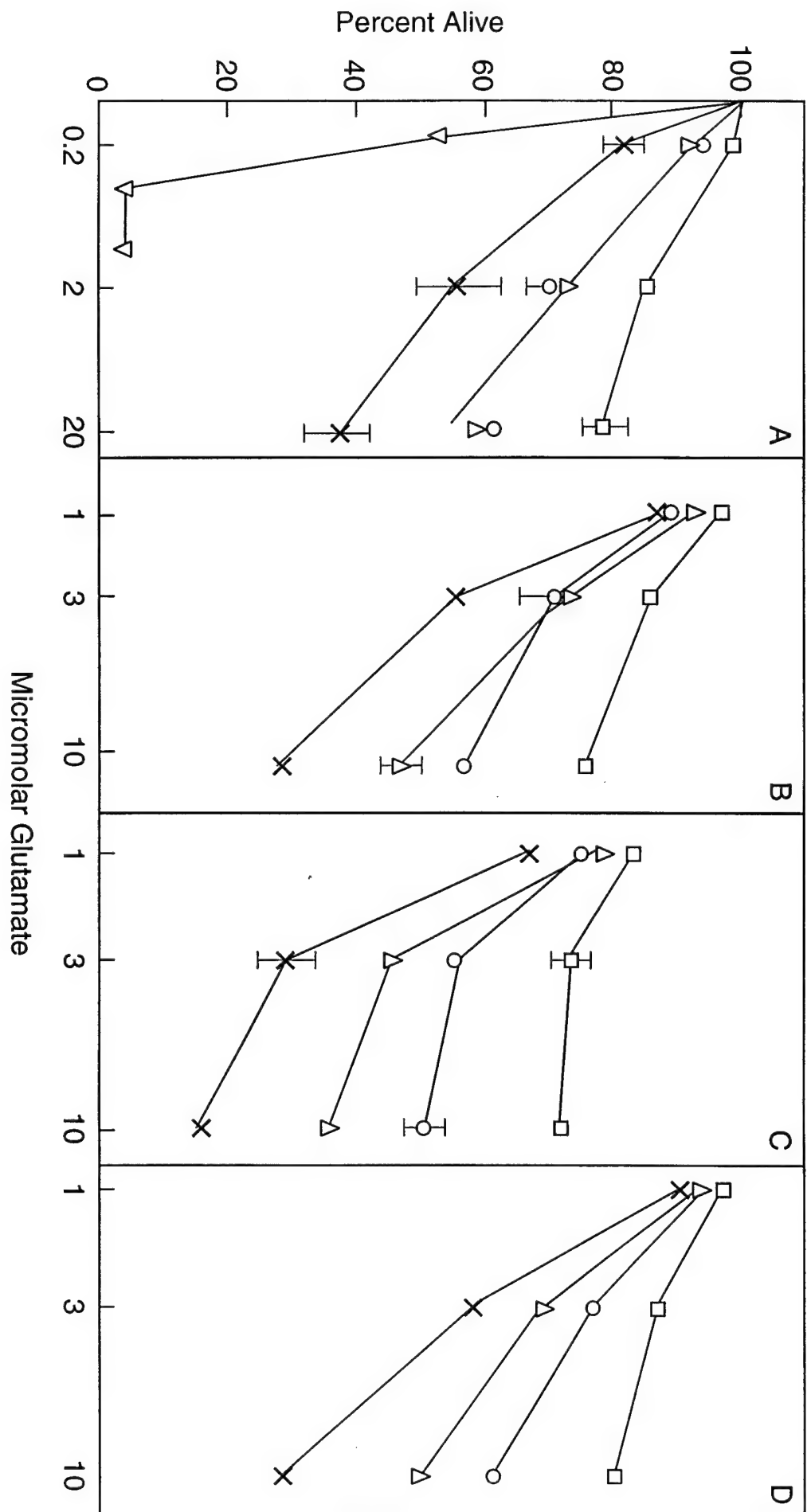


FIGURE 6

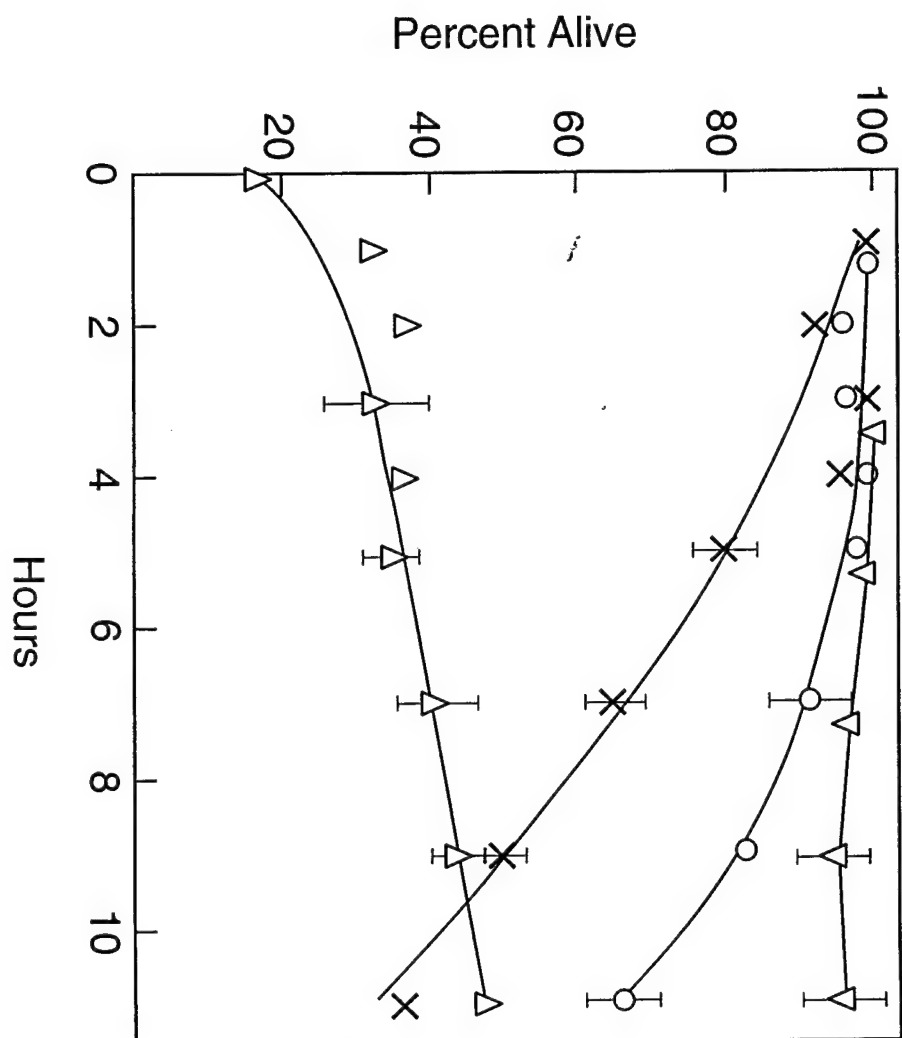


FIGURE 7

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## Regulation of Neuronal Antioxidant Metabolism by Translation Initiation Factor - 2 Alpha

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## Summary

Oxidative stress and highly specific decreases in glutathione (GSH) are associated with nerve cell death in Parkinson's disease. Using an experimental nerve cell model for oxidative stress and an expression cloning strategy, a gene involved in oxidative stress-induced programmed cell death was identified which both mediates the cell death program and regulates GSH levels. Two stress-resistant clones were isolated which contain antisense gene fragments of the translation initiation factor eIF2 $\alpha$  and express a low amount of eIF2 $\alpha$ . Sensitivity is restored when the clones are transfected with full length eIF2 $\alpha$ ; transfection of wild-type cells with the truncated eIF2 $\alpha$  gene confers resistance. The phosphorylation of eIF2 $\alpha$  also results in resistance to oxidative stress. In wild-type cells oxidative stress results in rapid glutathione depletion, a large increase in peroxide levels, and an influx of Ca<sup>2+</sup>. In contrast, the resistant clones maintain high glutathione levels and show no elevation in peroxides or Ca<sup>2+</sup> when stressed, and the glutathione synthetic enzyme gamma-glutamyl cysteine synthetase ( $\gamma$ GCS) is elevated. The change in  $\gamma$ GCS is regulated by a translational mechanism. eIF2 $\alpha$  is therefore a critical regulatory factor in the response of nerve cells to oxidative stress and in the control of the major intracellular antioxidant, GSH, and may play a central role in the many neurodegenerative diseases associated with oxidative stress.

## Introduction

Although programmed cell death (PCD) is a widely used mechanism for sculpturing the developing nervous system, its inappropriate activation leads to premature nerve cell death in neuropathological disorders such as Alzheimer's disease (AD) (Yankner, 1996) and Parkinson's disease (PD) (Mochizuki et al., 1996). Nerve cell death in both PD and AD are thought to be linked to oxidative stress, for antioxidant systems are upregulated and there is extensive evidence for excessive lipid and protein peroxidation (Jenner and Olanow, 1996; Simonian and Coyle, 1996). Associated with oxidative stress, there is an early and highly specific decrease in the glutathione content of the substantia nigra of PD patients (Perry et al., 1982; Sian et al., 1994; Sofic et al., 1992), which may precede the death of dopaminergic neurons (Dexter et al., 1994). In addition, the inhibition of  $\gamma$ -glutamyl-cysteine synthetase ( $\gamma$ GCS), the rate limiting step in GSH synthesis, results in the selective degeneration of dopaminergic neurons (Jenner and Olanow, 1996), and also potentiates the toxicity of 6 hydroxydopamine, MPTP and MPP<sup>+</sup>. These data suggest that GSH and oxidative stress play pivotal roles in the pathogenesis of AD and PD.

There are several ways in which the concentration of intracellular GSH and the oxidative burden of cells can be regulated. One of these is through extracellular glutamate. Although glutamate is generally thought of as both a neurotransmitter and an excitotoxin, extracellular glutamate can also kill neurons through a non-receptor mediated pathway which involves the glutamate-cystine antiporter, system Xc<sup>-</sup> (Bannai and Kitamura, 1980; Murphy et al., 1989; Sato et al., 1999). Under normal circumstances the concentration of extracellular cystine is high relative to intracellular cystine, and cystine is imported via the Xc<sup>-</sup> antiporter in exchange for intracellular glutamate. Cystine is ultimately converted to cysteine and utilized for protein synthesis and to make the antioxidant glutathione (GSH). However, when there is a high concentration of extracellular glutamate, the exchange of glutamate for cystine is

inhibited, and the cell becomes depleted of cysteine and GSH, resulting in severe oxidative stress. The cell eventually dies via a series of events which include the depletion of GSH, a requirement for macromolecular synthesis and caspase activity, lipoxygenase (LOX) activation, soluble guanylate cyclase activation, reactive oxygen species (ROS) accumulation, and finally  $\text{Ca}^{2+}$  influx (Li et al., 1997a; b; Murphy, et al., 1989; Tan et al., 1998a; b).

Programmed cell death caused by oxidative glutamate toxicity has characteristics of both apoptosis and necrosis (Tan et al., 1998b), and has been well studied in primary neuronal cell cultures (Murphy and Baraban, 1990; Oka et al., 1993), neuronal cell lines (Miyamoto et al., 1989; Murphy et al., 1989), tissue slices (Vornov and Coyle, 1991), and in the immortalized mouse hippocampal cell line, HT22 (Li et al., 1997a; b; Tan et al., 1998a; b). HT22 cells lack ionotropic glutamate receptors but die within 24 hours after exposure to 1-5 mM glutamate. Although the biochemical events have been well studied, little has been done to identify the transcriptional/translational changes which contribute to the glutamate-induced pathway of programmed cell death. Changes in gene expression clearly play a role in the cell death cascade since macromolecular synthesis is required early in the death pathway (Tan et al., 1998a; b). Through the use of a genetic screen, we identified the alpha subunit of the translation initiation factor 2 (eIF2 $\alpha$ ) as a gene whose expression is involved in oxidative stress-induced cell death and the regulation of intracellular GSH.

eIF2 is a trimeric complex involved in the initiation of translation (Hershey, 1991; Pain, 1996). The complex is made up of 3 subunits designated alpha, beta, and gamma, and behaves in a manner analogous to the trimeric G coupled proteins. The alpha subunit dictates whether protein synthesis will or will not take place and is often referred to as the control point for protein synthesis. The eIF2 complex brings the 40S ribosomal subunit together with the initiating tRNA<sub>met</sub> when eIF2 $\alpha$  is bound to GTP. Upon hydrolysis of GTP to GDP, the complex is no longer active and protein synthesis

is not initiated. GDP/GTP exchange takes place readily with the assistance of a guanine nucleotide exchange factor, eIF2B. However, when the alpha subunit of eIF2 is phosphorylated on serine 51, a change in the conformation enables it to bind and sequester eIF2B, thus inhibiting GDP/GTP exchange and protein synthesis.

eIF2 $\alpha$  phosphorylation takes place during ischemia (Burda et al., 1998; DeGracia et al., 1997), apoptosis (Sato et al., 1999; Srivastava et al., 1998), viral infection (Samuel, 1993; Wek, 1994), and following Ca<sup>2+</sup> influx (Prostko et al., 1995; Reilly et al., 1998; Srivastava et al., 1995). Therefore, eIF2 $\alpha$  may have significant roles in the cell death process following oxidative stress that are separate from its known function as a regulator of protein synthesis. The experiments described below show that the down-regulation or phosphorylation of eIF2 $\alpha$  protects nerve cells from oxidative stress-induced cell death by inhibiting GSH depletion and the increase in both ROS and intracellular Ca<sup>2+</sup> that are normally seen in cells exposed to oxidative stress. These data demonstrate a unique role of eIF2 $\alpha$  in oxidative stress induced programmed nerve cell death, acting as a translational switch which dictates whether a cell activates a survival response or follows a cell death pathway. eIF2 $\alpha$  may therefore play a central role in neuropathologies involving nerve cell death which are associated with oxidative stress.

## Results

### eIF2 $\alpha$ is Involved in the Oxidative Glutamate Toxicity Pathway

Although a mechanistic outline of oxidative glutamate toxicity mediated programmed cell death has been developed (Li et al., 1997a; b; Tan et al., 1998a; b), very little is known about the changes in gene expression that are required for this pathway. To identify genes that may be involved in cell death or the protection from cell death, HT22 cells were infected with a cDNA expression library in a retroviral

vector, and cells resistant to high concentrations of glutamate were selected. The retroviral library contained sense, antisense, and partial cDNA sequences. Therefore glutamate resistance could be due to a sense cDNA which, when over-expressed, causes glutamate resistance. Alternatively, a transcript from an antisense cDNA could interfere with the expression of a gene normally required for cell death, or the product of a partial cDNA fragment may act in a dominant-negative manner to block protein function. A fourth alternative is that during retroviral infection a cDNA is inserted into the genome in a way that disrupts or upregulates the normal expression of a gene that is involved in glutamate-induced cell death. Finally, ploidy sometimes changes in the cells as they divide, and resistant cells may arise independently of the retroviral infection due to loss of chromosomes or chromosome fragments. This constitutes a background level of naturally resistant clones in the genetic screen.

HT22 cells were infected with the retroviral cDNA library and selected in 10 mM glutamate for 48 hours, a condition where all of the cells normally die. The cDNA inserts in the pool of glutamate resistant cells were rescued by re-mobilizing the vector (Miller et al., 1993), and a second round of infection and selection identified 12 genes that play a putative role in oxidative glutamate toxicity. Identical fragments (213 base pairs) of the gene encoding the alpha subunit of the translation initiation factor - 2 (eIF2 $\alpha$ ) were identified in two separate clones. This gene was chosen for further study because of the requirement for protein synthesis in this form of cell death (Tan et al., 1998a; b). The subclones of HT22, designated clones 8 and 15, are extremely resistant to 10 mM glutamate (Fig. 1A). These clones are also resistant to other forms of oxidative stress including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and tert-butyl hydroperoxide, but not to cell death inducers such as TNF- $\alpha$ , anti-FAS antibody, serum starvation, and glucose deprivation (data not shown).



### Clones 8 and 15 cause Glutamate Resistance by Lowering eIF2 $\alpha$ Expression

As outlined previously, the introduction of the eIF2 $\alpha$  gene fragment into clones 8 and 15 with the retroviral cDNA library could lead to stress resistance by one of several mechanisms. It is unlikely that the eIF2 $\alpha$  gene fragment is causing glutamate resistance by disrupting or upregulating a gene whose expression is involved in cell death because the same sequence generates glutamate resistance upon re-infection. This leaves the possibility that the eIF2 $\alpha$  cDNA fragment is altering eIF2 $\alpha$  expression. Therefore the two resistant clones and wild-type cells were assayed for eIF2 $\alpha$  expression by western blotting. Although the antibody used for these studies can identify the phosphorylated form of eIF2 $\alpha$  (DeGracia et al, 1997), it recognizes both the dephosphorylated and phosphorylated forms of eIF2 $\alpha$  in HT22 cells (see Methods). Using this antibody, it was found that both clones 8 and 15 express lower levels of eIF2 $\alpha$  protein (Fig. 1B,C). Similar results were obtained with another antibody against eIF2 $\alpha$  (Ernst et al., 1987).

Since the retroviral expression library contained cDNAs in both the sense and antisense orientations, as well as partial fragments of cDNAs, it is likely that an antisense fragment was expressed to down regulate eIF2 $\alpha$  expression. The gene fragments that were rescued from clones 8 and 15 are identical and contain a fragment of the eIF2 $\alpha$  cDNA from the 3' end of the full sequence (728bp-941bp).

If the down-regulation of eIF2 $\alpha$  in the resistant clones is responsible for the resistance of the cells to glutamate, then the expression of full length eIF2 $\alpha$  should restore the sensitivity to glutamate. Transfection of full length eIF2 $\alpha$  human cDNA into both clones 8 and 15 restored glutamate sensitivity to both of the clones, while the empty vector had no effect (Figs. 2B and 2C). The restoration of glutamate sensitivity is not, however, up to the level of wild-type cells at the highest glutamate concentrations, probably because it was only possible to elevate eIF2 $\alpha$  to 80-90% of its original level (Fig. 1B and C). Wild-type HT22 cells remained sensitive to glutamate

after being transfected with the full length eIF2 $\alpha$  cDNA (Fig. 2A). This demonstrates that modulation of eIF2 $\alpha$  expression has significant effects on glutamate toxicity in HT22 cells.

### **eIF2 $\alpha$ Phosphorylation also Mediates Glutamate Resistance**

In order to confirm that the loss of eIF2 $\alpha$  activity is linked to glutamate resistance, a second method was employed which utilizes a dominant-negative approach to regulate eIF2 $\alpha$  function. The phosphorylated form of eIF2 $\alpha$  sequesters the guanine nucleotide exchange factor (eIF2B), resulting in a decrease in protein translation (Ernst et al., 1987). The S51D mutant of eIF2 $\alpha$  mimics constitutive phosphorylation when serine 51 in eIF2 $\alpha$  is replaced with an aspartic acid (Kaufman et al., 1989). The S51A mutant cannot be phosphorylated when serine 51 in eIF2 $\alpha$  is replaced with alanine (Pathak et al., 1988). Thus the S51D mutant inhibits protein synthesis while the S51A mutant prevents the shutdown of protein translation by the phosphorylation of eIF2 $\alpha$ . To assay the effect of eIF2 $\alpha$  phosphorylation on glutamate sensitivity, wild-type HT22 cells were infected with virus that contained either the S51D or S51A mutant, or an empty vector, and the cells were tested for glutamate resistance. HT22 cells infected with virus containing the mutant S51D become more resistant to glutamate (Fig. 3). The S51A mutant of eIF2 $\alpha$  did not have any effect on the response of the cells to glutamate relative to empty vector (Fig. 3). These data show that the down-regulation of eIF2 $\alpha$  activity by protein phosphorylation can lead to glutamate resistance and that eIF2 $\alpha$  phosphorylation may play an important role in cell death or survival after glutamate exposure. We could not, however, directly assay eIF2 $\alpha$  phosphorylation following glutamate exposure because none of the available antibodies immunoprecipitate or distinguish phosphorylated from unphosphorylated eIF2 $\alpha$  in HT22 cells.

### Changes in eIF2 $\alpha$ Expression do not Affect Translation Rates but do Slow Growth

To determine if eIF2 $\alpha$  down-regulation in the glutamate resistant clones causes a decrease in protein synthesis, protein translation rates were measured in clones 8 and 15 as well as in cells expressing mutants S51A and S51D. By inhibiting translation with cycloheximide, HT22 cells are able to survive in the presence of glutamate for short periods of time (Tan et al., 1998a; b). Therefore it was important to determine if the inhibition of translation is the sole mechanism by which clones 8, 15, and the S51D mutant expressing cell line become resistant to oxidative stress. To measure the rate of translation, cells were labeled with  $^3\text{H}$ -leucine for 30 minutes and the total cpm of incorporated leucine per mg protein calculated. The rate of translation in clone 15 is the same as in wild-type HT22 cells, but it is reduced about two fold in clone 8 (Fig. 4A). The rate of protein translation is unchanged in HT22 cells after infection with retrovirus containing the eIF2 $\alpha$  mutants (S51A and S51D) or empty vector (Fig. 4B). Similarly, exposure of HT22 cells to glutamate during a 10 hour time course does not lead to any significant changes in over-all protein translation (data not shown). These data indicate that the inhibition of overall protein synthesis is not the mechanism underlying protection by eIF2 $\alpha$ .

The translation rates do not, however, reflect the growth rates for each clone, for the growth rate of the wild-type HT22 cell line is more than 2 fold faster than either clone 8 or 15 (Fig. 4C). HT22 cells infected with the eIF2 $\alpha$  mutant S51D also have a slower growth rate than wild-type HT22 cells (Fig. 4D), even though the protein translation rate of this mutant is the same as that in the wild-type cells (Fig. 4B). In contrast, the S51A mutant has no significant effect on the translation rate (Fig. 4B) or the growth rate (Fig. 4D). These data show that changes in eIF2 $\alpha$  expression or activation by phosphorylation may lead to alterations in cell growth but not necessarily translation rates. However, it is possible that although the bulk of protein

synthesis is not altered, the synthesis of specific proteins required for cell proliferation and cell death is regulated by altered eIF2 $\alpha$  expression or phosphorylation.

### **eIF2 $\alpha$ Expression Alters Glutathione, ROS and Ca<sup>2+</sup> Responses to Glutamate**

To understand the role of eIF2 $\alpha$  in oxidative glutamate toxicity, several parameters of the glutamate response were measured in the resistant clones and the S51A and S51D mutant expressing cell lines and compared with the wild-type HT22 cells. HT22 cells undergo a rapid depletion of glutathione (GSH) upon exposure to glutamate (Tan et al., 1998a). After 8 hours of exposure to glutamate, GSH levels drop below 20% of their normal levels. Comparison of wild-type HT22 cells to glutamate resistant clones 8 and 15 after 10 hours of exposure to 5 mM glutamate revealed that the GSH levels in the resistant cells do not go below 50% of the GSH levels in untreated resistant clones. Furthermore, before glutamate exposure, both cell lines have higher GSH levels than untreated wild-type HT22 cells (Fig. 5A). The maximal difference in survival between the S51D mutant expressing cell line and the control HT22 cells is detected at 2 mM glutamate (Fig. 3). When GSH levels in wild-type cells infected with the S51A or S51D mutants, or the empty vector, are measured after 10 hours exposure to 2 mM glutamate, the S51D mutant cell line shows a decrease to about 50% of the original level compared with the 70% decrease in the wild-type, and empty vector infected cells. On the other hand, the S51A mutant cell line shows a decrease in GSH to about 20% of control levels (Fig. 5B). This pattern of GSH depletion is consistent with the survival data which demonstrate that while the S51D expressing HT22 cells are still healthy and dividing after 24 hours of glutamate exposure, the other cell lines are dead (Fig. 3).

HT22 cells exposed to glutamate for 10 hours show a very large increase in reactive oxygen species (ROS) which follows the drop in GSH (Tan et al., 1998a). The fluorescent dye, dichlorofluorescein (DCF), was used to determine the levels of ROS

production by flow cytometry in the resistant and mutant cell lines after exposure to toxic levels of glutamate. The level of ROS in wild-type HT22 cells after exposure to 5 mM glutamate for 10 hours is increased over 70 fold (Fig. 6A). In contrast, the glutamate resistant cell lines 8 and 15 do not show an increase in ROS above normal levels and the cells survive and continue to divide (Fig. 6A). When HT22 cells are exposed to 2 mM glutamate for 10 hours, the cells die and there is an increase in ROS, although the DCF intensity is more diffuse than with 5mM glutamate (Fig. 6B). The same pattern of increased DCF is seen in cells expressing empty vector and the mutant S51A. However, HT22 cells expressing the mutant S51D have low levels of ROS and were able to survive glutamate treatment (Fig. 6B).

Finally,  $\text{Ca}^{2+}$  influx was measured in wild-type, resistant, and phosphorylation mutant expressing cells.  $\text{Ca}^{2+}$  levels were determined by FACS analysis using the ratiometric dye Indo-1 (Tan et al., 1998a). After 10 hours exposure to 5 mM glutamate, HT22 cells have much higher levels of  $\text{Ca}^{2+}$  than untreated controls, while resistant cell lines 8 and 15 maintained intracellular  $\text{Ca}^{2+}$  levels similar to those of the wild-type HT22 cells (Fig. 6C). The same experiment was performed on the phosphorylation mutant expressing cell lines exposed to 2mM glutamate.  $\text{Ca}^{2+}$  levels increase significantly in the wild-type HT22 cells as well as the empty vector and S51A expressing HT22 cells. The intracellular  $\text{Ca}^{2+}$  level in the S51D expressing cells remained similar to the HT22 cells that were not exposed to glutamate (Fig. 6D). These data show that both the down-regulation of eIF2 $\alpha$  in clones 8 and 15 and the expression of the dominant-negative phosphorylation mutant S51D all prevent the decrease in GSH and the increases in ROS and  $\text{Ca}^{2+}$  normally associated with oxidative stress induced cell death.

### **The Inactivation of eIF2 $\alpha$ Upregulates Gamma-Glutamyl Cysteine Synthetase Expression by a Translational Mechanism**

Resistant clones 8 and 15 have decreased eIF2 $\alpha$  activity and increased basal levels of GSH. Furthermore, the resistant clones and the cells expressing the phosphorylation mutant, S51D, maintain GSH levels 50% above their basal levels after glutamate exposure. In order to determine if there is a causal relationship between eIF2 $\alpha$  protein levels and GSH production, expression of the rate-limiting enzyme for GSH synthesis,  $\gamma$ GCS, was examined in the wild-type cells and the resistant clones. Protein expression and mRNA levels of the catalytic subunit of  $\gamma$ GCS were measured by western and northern blotting respectively. Western blotting shows that the level of the catalytic subunit of  $\gamma$ GCS is 3 fold higher in the resistant clones than in the wild-type HT22 cells (Fig. 7A and C). In contrast, when both  $\gamma$ GCS and actin mRNA were quantitated and their ratio normalized to cells expressing the empty pCLBACE retroviral vector, the amount of  $\gamma$ GCS mRNA remained relatively constant (Fig. 7A and C). To rule out the possibility that eIF2 $\alpha$  activity changes the rate of  $\gamma$ GCS breakdown, resistant clone 15 and wild-type cells were treated with cycloheximide and the rate of protein loss followed by western blotting. This method gives values of protein turnover identical to pulse-chase experiments (Soucek et al., 1998). The rapidly turned over cell cycle protein, P27, served as a positive control (Soucek et al., 1998). Figure 7D shows that in contrast to P27,  $\gamma$ GCS was degraded more slowly but at the same rate in resistant and wild-type cells. These results indicate that a decrease in eIF2 $\alpha$  wild-type protein levels leads to an increase in production of the catalytic subunit of  $\gamma$ GCS by a translational mechanism, resulting in significantly higher levels of GSH.

If eIF2 $\alpha$  directly regulates  $\gamma$ GCS expression, then its expression should be up regulated in cells made resistant by the S51D phosphorylation mutant and down regulated in the resistant cells which were transfected with wild-type eIF2 $\alpha$  to render them more sensitive to oxidative stress. The levels of  $\gamma$ GCS increased about 60% in

S51D cells relative to wild-type cells, while  $\gamma$ GCS expression decreased between 20-40% in the resistant clones 8 and 15 (Fig. 7B). These data, along with those presented above, strongly suggest the eIF2 $\alpha$  expression and activity can directly modulate  $\gamma$ GCS protein levels. It is also likely, however, that the expression of additional proteins involved in the resistance to oxidative stress are regulated by eIF2 $\alpha$ .

## Discussion

The above data show that translation initiation factor 2 $\alpha$  plays a central role in programmed nerve cell death initiated by oxidative stress. Alterations in either the level of eIF2 $\alpha$  or its phosphorylation protect cells from glutamate-induced oxidative stress as well as other pro-oxidant agents. We will first discuss the evidence for the involvement of eIF2 $\alpha$  in glutamate-induced cell death, followed by possible mechanisms that eIF2 $\alpha$  could use to signal this type of cell death. The potential relevance of eIF2 $\alpha$  nerve cell death in Parkinson's disease will also be discussed.

### eIF2 $\alpha$ is Specifically Involved in Oxidative Glutamate Toxicity

HT22 glutamate resistant clones 8 and 15 were derived from a genetic screen after infection with a retrovirus based cDNA expression library and selection with a high concentration of the pro-oxidant glutamate. Both clones contain an identical fragment of the gene for eIF2 $\alpha$  from the retroviral library. The following evidence shows that eIF2 $\alpha$  activity is required for cells to die via oxidative glutamate toxicity and other forms of oxidative stress: 1) eIF2 $\alpha$  fragments rescued from the glutamate resistant cells make wild-type cells resistant to glutamate upon re-infection. 2) Western blotting demonstrates that the eIF2 $\alpha$  protein levels in the resistant clones are lower than in wild-type HT22 cells. 3) eIF2 $\alpha$  down-regulation alone causes resistance to glutamate, since clones 8 and 15, when transfected with full length human eIF2 $\alpha$ , become glutamate sensitive.

Since eIF2 $\alpha$  regulates the rate of protein translation, and cell death requires protein synthesis, it is possible that the inhibition of cell death simply reflects a decrease in the rate of protein synthesis in the resistant cells. However, the decrease of eIF2 $\alpha$  in the resistant cells did not necessarily lead to a slower rate of protein synthesis. Although clones 8 and 15 are equally resistant to glutamate, only clone 8 has a rate of protein synthesis which is lower than that in the wild-type cells. In addition, cells infected with the eIF2 $\alpha$  phosphorylation mutant S51D, which also induces glutamate resistance, synthesize protein at a rate that is equal to that of the wild-type cells. These results indicate that a decrease in the rate of translation *per se* does not lead to glutamate resistance.

Further evidence that eIF2 $\alpha$  phosphorylation plays a key role in determining the fate of the glutamate-exposed HT22 cells is evident when the S51D mutant of eIF2 $\alpha$  is expressed in the HT22 cells, resulting in glutamate resistance. The S51D mutant mimics a constitutively phosphorylated form of eIF2 $\alpha$  that cannot be dephosphorylated, such that it is able to sequester the guanine nucleotide exchange factor eIF2B and inhibit the initiation of protein synthesis (Ernst et al., 1987; Kaufman et al., 1989). Since the infection of HT22 cells with either eIF2 $\alpha$  or the phosphorylation mutants leads to over-expression of their respective transcripts but does not alter the overall levels of eIF2 $\alpha$  protein (data not shown), the amount of eIF2 $\alpha$  protein that is synthesized must be highly regulated. In contrast to our data, the S51D mutant causes apoptosis when transiently transfected into another cell line (Srivastava, et al., 1998), presumably because it shuts down protein synthesis. However, in the HT22 cells expressing the S51D mutant, the cells maintain a normal protein synthesis rate, although the growth rate is slower than in the wild-type cells (Fig. 4D).

One explanation for how the HT22 cells infected with the S51D mutant are able to maintain reasonable translation and growth rates is that after infection with the S51D mutant, cells that greatly over-express the mutant die, while the cells that mildly



over-express the mutant protein are able to survive at a slightly slower growth rate. This is likely because the infected cells become less resistant to glutamate with time. Therefore, they probably express sufficient amounts of the S51D mutant to survive glutamate exposure, but the cells that express the lower amounts of the mutant insufficient for survival in glutamate are eventually able to outgrow the other cells when not in the presence of glutamate.

### **eIF2 $\alpha$ Down-Regulation and the Constitutively Phosphorylated Form of eIF2 $\alpha$ Alter the Same Intermediates in the Cell Death Pathway**

The observation that the two glutamate resistant clones selected by expression cloning and the over-expression of the phosphorylation mutant, S51D, produce similar changes in cell physiology during glutamate exposure further supports the critical role of eIF2 $\alpha$  in the toxicity cascade. These cell lines all exhibit higher GSH levels than controls following glutamate exposure, and lower levels of ROS and intracellular Ca<sup>2+</sup>. GSH levels in wild-type HT22 cells decline to below 20% of controls following glutamate exposure, while GSH levels in both the resistant clones and the cells expressing the dominant-negative S51D mutant drop to less than 50% of their basal levels. In contrast to control levels, this level of GSH is sufficient to maintain cell viability (see also, Sagara and Schubert, 1998). The basal levels of GSH in the resistant clones were also higher than in the wild-type HT22 cells. It could be argued that the lower rate of translation and cell growth in the resistant cells frees up more cysteine, allowing them to maintain a higher basal level of GSH. However, clone 15 has a very high basal level of GSH, but a normal rate of protein synthesis, suggesting that the resistant cells have higher GSH levels because they actively produce greater amounts of this antioxidant.

The above results suggest that the down-regulation or phosphorylation of eIF2 $\alpha$  during times of stress signals the translation of specific proteins that increase cell

survival. Since decreases in either eIF2 $\alpha$  activity or protein levels both lead to an increase in GSH, we asked if the rate limiting enzyme in GSH production,  $\gamma$ GCS, was increased in the resistant cells compared with the wild-type HT22 cells. Figure 7 shows that while the amount of  $\gamma$ GCS is increased in the original resistant clones, the  $\gamma$ GCS mRNA level remains constant, and there is no difference in the rates of  $\gamma$ GCS breakdown. In addition,  $\gamma$ GCS is upregulated by the phosphorylation mutant S51D, and down-regulated by the introduction of additional eIF2 $\alpha$  into the glutamate resistant clones 8 and 15 (Fig. 7). These data show that eIF2 $\alpha$  regulates  $\gamma$ GCS expression by a translational mechanism. Amino acid starvation in *Saccharomyces cerevisiae* also causes eIF2 $\alpha$  phosphorylation and leads to the selective translation of one specific transcription factor that signals the synthesis of amino acids so that the yeast can survive starvation (Samuel, 1993). A mechanism comparable to that employed by the yeast may be utilized in HT22 cells when eIF2 $\alpha$  activity is low, leading to an increased production of  $\gamma$ GCS to promote cell survival.

### **eIF2 $\alpha$ Plays a Unique Role in Programmed Cell Death**

There have been several reports that positively link eIF2 $\alpha$  to apoptosis. eIF2 $\alpha$  phosphorylation by PKR kinase is the cause of cell death in TNF- $\alpha$  stimulated cells (Srivastava, et al., 1998), and eIF2 $\alpha$  is cleaved by caspases following an increase in PKR kinase activity induced by TNF- $\alpha$  or poly (I):poly (C) (Sato et al., 1999). HT22 resistant clones 8 and 15 are, however, not resistant to TNF- $\alpha$ , indicating that they utilize a survival mechanism that is unique to oxidative stress. Ischemia and reperfusion in the rat brain also leads to eIF2 $\alpha$  phosphorylation and cell death (Burda et al., 1998; DeGracia et al., 1997). The common denominator in all three forms of cell death is the phosphorylation of eIF2 $\alpha$ . In these cases, death signals lead to eIF2 $\alpha$  phosphorylation, protein synthesis shutdown, and cell lysis. In contrast, our data and at least one other study, show that eIF2 $\alpha$  phosphorylation is able to protect cells from death. Cells

treated with type I interferons induce eIF2 $\alpha$  phosphorylation, enabling the cells to survive infection by a wide array of viruses by temporarily shutting down protein synthesis to prevent viral replication. In addition, HT22 cells treated with thapsigargin, a substance shown to cause eIF2 $\alpha$  phosphorylation (Prostko et al., 1995), leads to cell survival following glutamate exposure (data not shown). It is therefore likely that the extent of eIF2 $\alpha$  phosphorylation controls the cellular response to a given stimulus. Likewise, the nature of the stimulus determines whether eIF2 $\alpha$  phosphorylation will be utilized to prevent or promote cell death.

The above experiments link oxidative stress, GSH depletion, and the regulation of  $\gamma$ GCS directly to eIF2 $\alpha$  and programmed nerve cell death. Markers for both oxidative stress and the depletion of intracellular GSH are found in areas of CNS nerve cell death in Parkinson's disease (Sian et al., 1994). In both Parkinson's and Alzheimer's diseases, large numbers of nerve cells do, however, survive. It is therefore important to understand the mechanisms which lead to resistance to oxidative stress. In the brain, intracellular GSH is the single most important antioxidant, and GSH-peroxidase breaks down H<sub>2</sub>O<sub>2</sub> and a variety of organic peroxides, thus protecting cells from oxidative stress. The experiments presented here show that changes in the expression level or phosphorylation of a member of the protein translation complex, eIF2 $\alpha$ , can regulate the ability of a nerve cell to deal with oxidative stress. This appears to be primarily done through the regulation of GSH levels, for sustained GSH depletion is the initial event which triggers downstream events such as peroxide accumulation and ultimately cell death. Cells with low amounts of eIF2 $\alpha$  or phosphorylated eIF2 $\alpha$  maintain high levels of GSH when stressed and do not die. These results point to a central role of eIF2 $\alpha$  as a translational switch in the control of oxidative stress within the nervous system. They also suggest a possible therapeutic target for manipulating intracellular GSH levels.

## Experimental Procedures

The following chemicals were purchased from Sigma Chemical Company (St. Louis, MO): puromycin, trichloroacetic acid (TCA), formic acid, glutathione (GSH), glutathione reductase, triethanolamine, sulfosalicylic acid, NADPH, bovine serum albumin fraction V (BSA), glutaraldehyde, L-glutamic acid (glutamate). The fluorescent probes 2',7'-dichlorofluorescein diacetate (DCF) and indo-acetoxymethylester (Indo-1), pluronic F-127, and propidium iodide were obtained from Molecular Probes (Eugene, OR). The Coomassie Plus Protein Assay Reagent and the Super Signal Substrate were both purchased from Pierce (Rockford, IL). Immobilon-P was purchased from Millipore (Bedford, MA).

## Infection with the Retroviral cDNA Library

HT22 cells were infected with the retroviral vector pcLXSN containing a cDNA library derived from the human embryonic lung cell line, MRC-5 (Somia et al., 1999). The library contained  $2 \times 10^6$  cDNAs, and the HT22 cells were infected with approximately  $10^7$  virus particles. The cDNA library contains both sense and antisense sequences. The retrovirus stably integrates into the host cell's genomic DNA and expresses the cDNA inserted between its long terminal repeats (LTR). Clones containing genes that confer glutamate resistance were identified by selecting cells that survived in 10 mM glutamate. Genomic DNA from each clone was analyzed by PCR using primers that straddle the cDNA insert in the retroviral vector. The cDNA inserts were then subcloned and sequenced.

Viral vectors were rescued from the clones by transfection with an ecotropic helper plasmid. These viral particles were collected from the media, and used to infect the packaging cell line, PA317, which amplified the virus (Miller et al., 1993). The viral medium from the packaging cells was then used to infect wild-type HT22 cells in order to confirm that the cDNA was indeed able to make the HT22 cells resistant to glutamate.

### Immunoblotting and Northern Procedures

Cells were plated at  $5 \times 10^5$  cells per 100 mm dish 12-16 hours prior to use, and lysed in sample buffer containing 3% SDS. Lysates were sonicated, protein concentrations were normalized using the Coomassie Plus protein assay reagent from Pierce (Rockford, IL), and 25  $\mu$ g protein was loaded per lane on 12% tris-glycine SDS-PAGE gels (Novex, San Diego, CA). Gels were transferred onto Immobilon-P membrane (Millipore, Bedford, MA), and blocked with 5% milk in tris buffered saline (TBS) for 1 hour at room temperature. An antibody against eIF2 $\alpha$  (Research Genetics, Huntsville, AL) was previously shown to recognize only phosphorylated eIF2 $\alpha$ . However, in our hands the antibody recognized both phosphorylated and unphosphorylated protein when the western blots and lysates were dephosphorylated with a mixture of bovine and calf intestine alkaline phosphatase. Blots were also probed with antibodies against both phosphorylated and total MAP kinase to confirm that proteins were completely dephosphorylated after treatment with the phosphatases. Therefore, this anti-eIF2 $\alpha$  antibody was used to determine the levels of total eIF2 $\alpha$  in the HT22 cell and the resistant clones 8 and 15. The anti-eIF2 $\alpha$  primary antibody was diluted into 5% bovine serum albumin (BSA) in TBS plus Tween20 (TTBS) at 1:250 and placed on the blot overnight at 4°C. Blots were incubated with the secondary antibody, goat anti-rabbit IgG HRP conjugated (BioRad), for 1 hour at room temperature at a dilution of 1:20,000 in 5% milk in TTBS. Blots were exposed to Kodak X-OMAT Blue film for chemiluminescence (Rochester, NY) using the SuperSignal substrate from Pierce (Rockford, IL).

Northern blots of the  $\gamma$ GCS catalytic subunit were done as described in the original manuscript in which cDNA clones were isolated (Gipp et al., 1992). Northern blots were done using a probe consisting of the carboxyl terminal 387 a of the protein, which detected a single band of about 3.7 kb.

### **Transfection of Full Length eIF2 $\alpha$ into Clones 8 and 15**

The full length cDNA for eIF2 $\alpha$  was obtained from Dr. Miyamoto (NHLBI, Bethesda, MD), and was cloned into the pCLBABEpuro retroviral vector, a modified version of the pBABEpuro vector (Morgenstern and Land, 1990). This vector was then used for transfection with lipofectamine (GibcoBRL).

### **Production of Retrovirus Expressing the Dominant-Negative Mutants of eIF2 $\alpha$**

The cDNA constructs for two mutants of eIF2 $\alpha$  (S51A and S51D) were obtained from Dr. Kaufman (University of Michigan, Ann Arbor, MI), and subcloned into pCLBABEpuro. Retroviral vectors were made as described (Somia et al., 1999), with either pCLBABE-S51A, pCLBABE-S51D, or pCLBABEpuro alone. The viral vectors were used to infect HT22 cells and infected cells were selected in 4  $\mu$ g/ml puromycin (Sigma, St. Louis, MO). The puromycin resistant cells were tested for glutamate resistance by the MTT cell death assay (Tan, et al., 1998a).

### **Translation and Degradation Assays**

For translation assays, cells were labeled in 60 mm dishes with 500,000 cpm of  $^3$ H-leucine diluted in DMEM supplemented with 10% fetal bovine serum for 30 minutes. The cells were then washed with ice cold serum free DMEM and lysed on the dish using 1 ml ice cold 10% trichloroacetic acid (TCA) plus 1 mM dithiothreitol (DTT) and 1 mM cold leucine. Cellular protein was precipitated, dissolved in formic acid, and the  $^3$ H-leucine incorporation determined by scintillation counting. The protein concentration was determined using the Coomassie Blue Plus protein reagent (Pierce). The total cpm  $^3$ H-leucine incorporated per mg protein for 30 minutes was calculated for each sample. Samples were prepared in triplicate. Protein degradation assays were

done exactly as described elsewhere (Soucek et al., 1998). Cells were treated with 100 $\mu$ g/ml cycloheximide and protein abundance followed by western blotting.

### **Growth Assays**

Five sets of triplicate dishes of cells were plated at  $5 \times 10^4$  in 35 mm dishes. The triplicate sets of each cell type were counted at 12, 24, 48, and 72 hours after plating. Cells were dissociated using 5x pancreatase (Gibco) for 15 minutes, resuspended in Dulbecco's Modified Eagle's Medium (DMEM), and placed in eppendorf tubes. Cells were counted directly on a Coulter Counter after dilution in isotonic saline. The data are plotted as cell counts versus time in order to compare the growth rates for the different clones.

### **Glutathione Assay**

Total intracellular reduced (GSH) and oxidized (GSSG) glutathione were measured as previously described (Tan et al., 1998a). Briefly, cells were plated on 60 mm tissue culture dishes at  $2 \times 10^5$  cells per dish 12 hours before adding 2-5 mM glutamate for 10 hours and total glutathione assayed. Pure GSH was used to establish a standard curve.

### **Flow Cytometric Studies**

Cells were plated on 60 mm dishes at  $2 \times 10^5$  cells per dish 12 hours before adding 2-5 mM glutamate for 10 hours. Samples were then labeled with the fluorescent dyes 2',7'-dichlorofluorescein diacetate (DCF) and Indo-1 acetoxymethylester (Indo-1) to determine reactive oxygen species (ROS) production and  $\text{Ca}^{2+}$  influx, respectively. Samples were prepared as described previously (Tan, et al., 1998a).

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## References

- Bannai, S. and Kitamura, E. (1980). Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. *J. Biol. Chem.* 255, 2372-2376.
- Burda, J., Martín, M., Gottlieb, M., Chavko, M., Marsala, J., Alcázar, A., Pavón, M., Fando, J. and Salinas, M. (1998). The intraischemic and early reperfusion changes of protein synthesis in the rat brain. eIF-2 $\alpha$  kinase activity and role of initiation factors eIF-2 $\alpha$  and eIF-4E. *J. Cereb. Blood Flow Metab.* 18, 59-66.
- DeGracia, D.J., Sullivan, J.M., Neumar, R.W., Alousi, S.S., Hikade, K.R., Pittman, J.E., White, B.C., Rafols, J.A. and Krause, G.S. (1997). Effect of brain ischemia and reperfusion on the localization of phosphorylated eukaryotic initiation factor 2 $\alpha$ . *J. Cereb. Blood Flow and Metab.* 17, 1291-1302.
- Dexter, D.T., Sian, J., Rose, S., Hindmarsh, J.G., Mann, V.M., Copper, J.M. and Wells, F.M., et al. (1994). Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. *Ann. Neurol.* 35, 38-44.
- Ernst, H., Duncan, R.F. and Hershey, J.W.B. (1987). Cloning and sequencing of complementary DNAs encoding the  $\alpha$ -subunit of translational initiation factor eIF-2. Characterization of the protein and its messenger RNA. *J. Biol. Chem.* 262, 1206-1212.
- Gipp, J.J., Chang, C. and Mulcahy, R.T. (1992). Cloning and nucleotide sequence of a full-length cDNA for human liver  $\gamma$ -glutamylcysteine synthetase. *Biochem. Biophys. Res. Comm.* 185, 29-35.

Hershey, J. (1991). Translational control in mammalian cells. *Annu. Rev. Biochem.* 60, 717-755.

Jenner, P. and Olanow, C.W. (1996). Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology* 47, s161-s170.

Kaufman, R.J., Davies, M.V., Pathak, V.K. and Hershey, J.W. (1989). The phosphorylation state of eucaryotic initiation factor 2 alters translational efficiency of specific mRNAs. *Mol. Cell Biol.* 9, 946-958.

Li, Y., Maher, P. and Schubert, D. (1997a). Requirement for cGMP in nerve cell death caused by glutathione depletion. *J. Cell Biol.* 139, 1317-24.

Li, Y., Maher, P. and Schubert, D. (1997b). A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. *Neuron* 19, 453-63.

Miller, A., Miller, D., Garcia, J. and Lynch, C. (1993). Use of retroviral vectors for gene transfer and expression. *Meth. Enzymol.* 217, 581-599.

Miyamoto, M., Murphy, T.H., Schnaar, R.L. and Coyle, J.T. (1989). Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line. *J. Pharmacol. Exp. Ther.* 250, 1132-40.

Mochizuki, H., Goto, K., Mori, H. and Mizuno, Y. (1996). Histochemical detection of apoptosis in Parkinson's disease. *J. Neurol. Sci.* 137, 120-123.

Morgenstern, J.P. and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18, 3587-96.

Murphy, T.H. and Baraban, J.M. (1990). Glutamate toxicity in immature cortical neurons precedes development of glutamate receptor currents. *Brain Res.* 57, 146-50.

Murphy, T.H., Miyamoto, M., Sastre, A., Schnaar, R.L. and Coyle, J.T. (1989). Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* 2, 1547-58.

Oka, A., Belliveau, M.J., Rosenberg, P.A. and Volpe, J.J. (1993). Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. *J. Neurosci.* 13, 1441-1453.

Pain, V. (1996). Initiation of protein synthesis in eukaryotic cells. *Eur. J. Biochem.* 236, 747-771.

Pathak, V.K., Nielsen, P.J., Trachsel, H. and Hershey, J.W. (1988). Structure of the beta subunit of translational initiation factor eIF-2. *Cell* 54, 633-639.

Perry, T.L., Godin, D.V. and Hansen, S. (1982). Parkinson's disease: a disorder due to nigral glutathione deficiency? *Neurosci. Lett.* 33, 305-310.

Prostko, C., Dholakia, J., Brostrom, M. and Brostrom, C. (1995). Activation of the double-stranded RNA-regulated protein kinase by depletion of endoplasmic reticular calcium stores. *J. Biol. Chem.* 270, 6211-6215.

Reilly, B., Brostrom, M. and Brostrom, C. (1998). Regulation of protein synthesis in ventricular myocytes by vasopressin. The role of sarcoplasmic/endoplasmic reticulum. *J. Biol. Chem.* 273, 3747-3755.

Sagara, Y. and Schubert, D. (1998). The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. *J. Neurosci.* 18, 6662-71.

Samuel, C. (1993). The eIF-2 $\alpha$  protein kinases, regulators of translation in eukaryotes from yeasts to humans. *J. Biol. Chem.* 269, 7603-7606.

Sato, H., Tamba, M., Ishii, T. and Bannai, S. (1999). Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J. Biol. Chem.* 274, 11455-11458.

Satoh, S., Hijikata, M., Handa, H. and Shimotohno, K. (1999). Caspase-mediated cleavage of eukaryotic translation initiation factor subunit 2 $\alpha$ . *Biochem. J.* 342, 65-70.

Sian, J., Dexter, D.T., Lees, A.J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P. and Marsden, C.D. (1994). Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann. Neurol.* 36, 348-355.

Simonian, N.A. and Coyle, J.T. (1996). Oxidative stress in neurodegenerative diseases. *Annu. Rev. Pharmacol. Toxicol.* 36, 83-106.

Sofic, E., Lange, K.W. and Riederer, P. (1992). Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neurosci. Lett.* 142, 128-130.

- Somia, N., Schmitt, M., Vetter, D., Van Antwerp, D., Heinemann, S. and Verma, I. (1999). LFG: an anti-apoptotic gene that provides protection from Fas-mediated cell death. *Proc. Natl. Acad. Sci. USA* 96, 12667-12672.
- Soucek, T., Yeung, R.S. and Hengstschlager, M. (1998). Inactivation of the cyclin-dependent kinase inhibitor p27 upon loss of the tuberous sclerosis complex gene-2. *Proc. Natl. Acad. Sci. USA* 95, 15653-15658.
- Srivastava, S., Davies, M. and Kaufman, R. (1995). Calcium depletion from the endoplasmic reticulum activates the double-stranded RNA-dependent protein kinase (PKR) to inhibit protein synthesis. *J. Biol. Chem.* 270, 16619-16624.
- Srivastava, S., Kumar, K. and Kaufman, R. (1998). Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. *J. Biol. Chem.* 273, 2416-2423.
- Tan, S., Sagara, Y., Liu, Y., Maher, P. and Schubert, D. (1998a). The regulation of reactive oxygen species production during programmed cell death. *J. Cell Biol.* 141, 1423-32.
- Tan, S., Wood, M. and Maher, P. (1998b). Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. *J. Neurochem.* 71, 95-105.
- Vornov, J.J. and Coyle, J.T. (1991). Glutamate neurotoxicity and the inhibition of protein synthesis in the hippocampal slice. *J. Neurochem.* 56, 996-1006.

Wek, R.C. (1994). eIF-2 kinases: regulators of general and gene-specific translation initiation. *Trends Biochem. Sci.* 19, 491-496.

Yankner, B.A. (1996). Mechanisms of neuronal degeneration in Alzheimer's disease. *Neuron* 16, 921-32.

## Figure Legends

### Figure 1. Clones 8 and 15 are Resistant to Glutamate and Express Low Levels of eIF2 $\alpha$

Glutamate resistant cells were cloned after infecting HT22 cells with a retroviral cDNA expression library.

(A) Cell viability was measured using the MTT assay with wild-type HT22 cells (open bars), clone 8 (black bars), and clone 15 (hatched bars) after a 24 hour exposure to 0, 5 mM, and 10 mM glutamate. Samples were measured in triplicate on 96 well plates (n=10).

(B and C) Glutamate resistant clones 8 and 15 have lower levels of eIF2 $\alpha$  protein than wild-type HT22 cells, which are largely restored by the reintroduction of wild-type eIF2 $\alpha$ . B) eIF2 $\alpha$  and actin protein levels were detected by western blotting of cell lysates (25 $\mu$ g) from wild-type HT22 cells, glutamate resistant clones 8 and 15, and clones 8 and 15 transfected with wild-type eIF2 $\alpha$ .

(C) The density of each protein band was measured using the program NIH Image, and the average density for each band was plotted relative to eIF2 $\alpha$  in wild-type HT22 cells. Identical amounts ( $\pm 5\%$ ) of actin in each lane served as loading controls. The experiment was repeated at least 5 times with similar results.

\*Significantly different from HT22 wild-type controls (mean  $\pm$  SEM,  $P < 0.05$ )

### Figure 2. Glutamate Resistant Clones 8 and 15 Acquire Glutamate Sensitivity Following Transfection with Full Length eIF2 $\alpha$

Wild-type HT22 cells and glutamate resistant clones 8 and 15 were stably transfected with an expression construct of eIF2 $\alpha$ .

(A) The wild-type HT22 cells were unaffected by transfection of eIF2 $\alpha$ .

(B and C) Resistant clones 8 and 15, when transfected with the eIF2 $\alpha$  construct, became glutamate sensitive as detected by the MTT assay after 24 hours of exposure to 2 mM, 5 mM, and 10 mM glutamate. Samples were measured in triplicate (n=3).

**Figure 3. HT22 Cells Become Glutamate Resistant When the eIF2 $\alpha$  S51D Mutant is Stably Expressed**

HT22 cells were infected with virus containing either the pCLBABEpuro empty vector (-◆-), the S51A mutant of eIF2 $\alpha$  (-○-), or the eIF2 $\alpha$  S51D mutant (-▲-). Cell viability was measured by the MTT assay. Samples were prepared in triplicate (n=4).

**Figure 4. Effect of eIF2 $\alpha$  Expression on the Rate of Protein Translation**

Protein synthesis was measured by <sup>3</sup>H-leucine incorporation for 30 minutes and normalized to total protein.

(A) Wild-type HT22 cells, cells exposed to 5 mM glutamate (Glu) for 4 hours, cycloheximide (Cx), and clones 8 and 15

(B) Wild-type HT22 cells and cells infected with either empty vector, or the eIF2 $\alpha$  phosphorylation mutants S51A or S51D. All assays were performed in triplicate (n=2). The growth rate is decreased when eIF2 $\alpha$  is down-regulated or phosphorylated in HT22 cells. Growth rates were measured by counting cells at 0, 24, 48, and 72 hours after plating.

(C) Wild-type HT22 cells (-■-); resistant clone 15 (-▲-); and resistant clone 8 (-◆-).

(D) HT22 cells (-■-); cells infected with empty vector (-◆-); eIF2 $\alpha$  mutant S51A (-○-); and mutant S51D (-▲-). Cell counts at all time points were done in triplicate, and the data are presented as the average, plus or minus the standard error of the mean.



### Figure 5. GSH levels in Resistant Cells

GSH levels were measured in control, untreated cells, and cells exposed to 5 mM glutamate or 2 mM glutamate for 10 hours.

(A) 5 mM glutamate. Clones 8 (clear bars) and 15 (hatched bars) have higher basal GSH levels than wild-type HT22 and only deplete to 72% $\pm$ 4 and 56% $\pm$ 1 of their basal GSH levels respectively, with glutamate exposure.

(B) 2 mM glutamate. Wild-type HT22 cells (solid bars), empty vector infected cells (clear bars), and S51A mutant infected cells (hatched bars) show GSH levels that are depleted to 20-30% of basal levels. In the S51D mutant expressing cells (narrow hatched bars), GSH depleted to only about 50% of the basal level. 100% GSH is defined as the GSH level assayed in the untreated control cells. The numbers above the glutamate exposed bars indicate the percentage of GSH relative to the basal level in the same cell line (n=3).

### Figure 6. ROS and Ca<sup>2+</sup> Levels Following Exposure to Glutamate

ROS levels were measured by flow cytometry using the fluorescent dye DCF.

(A) ROS levels increase 72 fold (gray line) in wild-type HT22 cells exposed to 5 mM glutamate for 10 hours. However, ROS levels do not increase in resistant clones 8 and 15 after 10 hours exposure to glutamate.

(B) ROS levels following exposure to glutamate are increased (gray line) in wild-type HT22 cells and in HT22 cells infected with empty vector or the S51A mutant of eIF2 $\alpha$ . ROS levels do not increase in HT22 cells expressing the eIF2 $\alpha$  mutant S51D when exposed to 2 mM glutamate for 10 hours. 10,000 live cells were assayed and the experiment was repeated 2 times with similar results.

(C) Cytosolic Ca<sup>2+</sup> levels were measured using flow cytometry and the ratiometric dye Indo-1. HT22 cells exposed to 5 mM glutamate for 10 hours have a large increase in cytosolic Ca<sup>2+</sup> compared to untreated cells. After glutamate exposure, resistant

clones 8 and 15 maintain cytosolic  $\text{Ca}^{2+}$  levels similar to the wild-type untreated control.

(D) HT22 cells exposed to 2 mM glutamate for 10 hours have a large increase in cytosolic  $\text{Ca}^{2+}$ . HT22 cells infected with the empty vector or the S51A mutant of eIF2 also show similar increases in  $\text{Ca}^{2+}$ . The S51D mutant of eIF2 $\alpha$  prevents the glutamate-induced increase in  $\text{Ca}^{2+}$  when stably expressed in HT22 cells. All samples were prepared in duplicate. 10,000 live cells were assayed in each experiment, and the study was repeated twice with similar results.

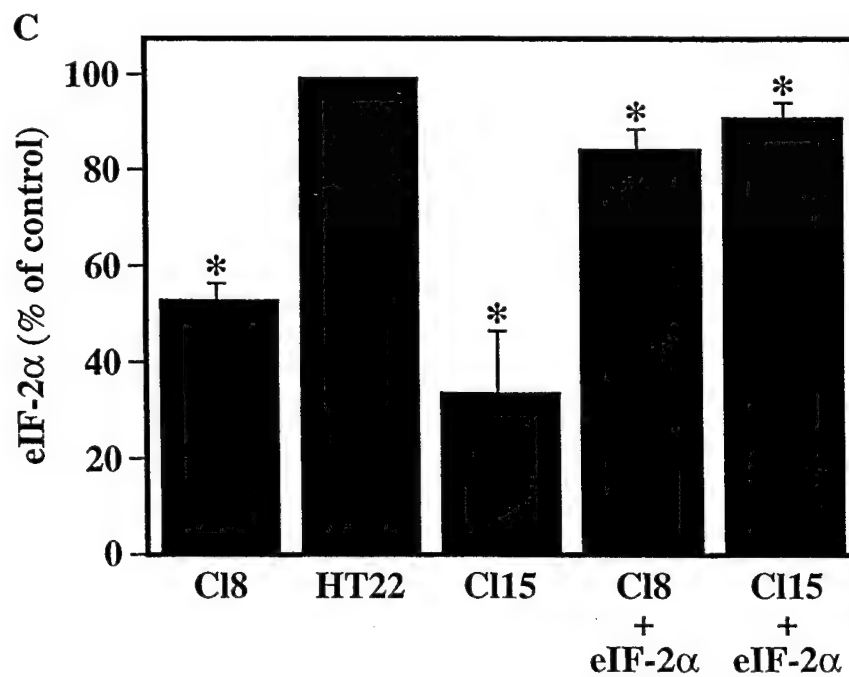
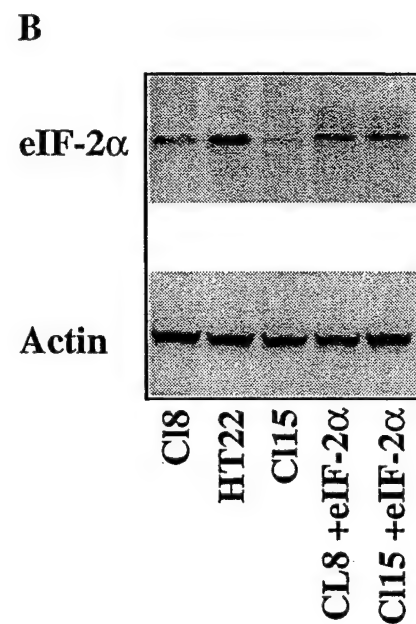
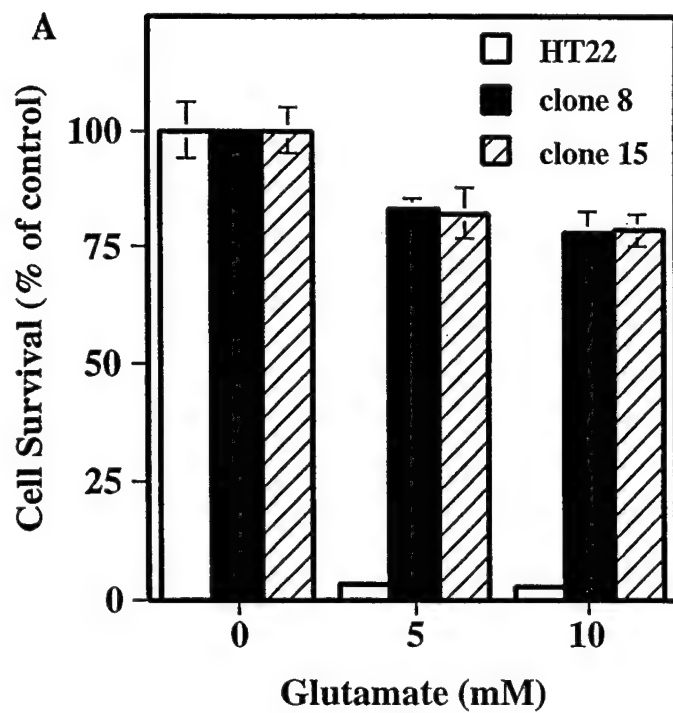
#### Figure 7. $\gamma$ GCS Protein Expression is Regulated at the Level of Translation

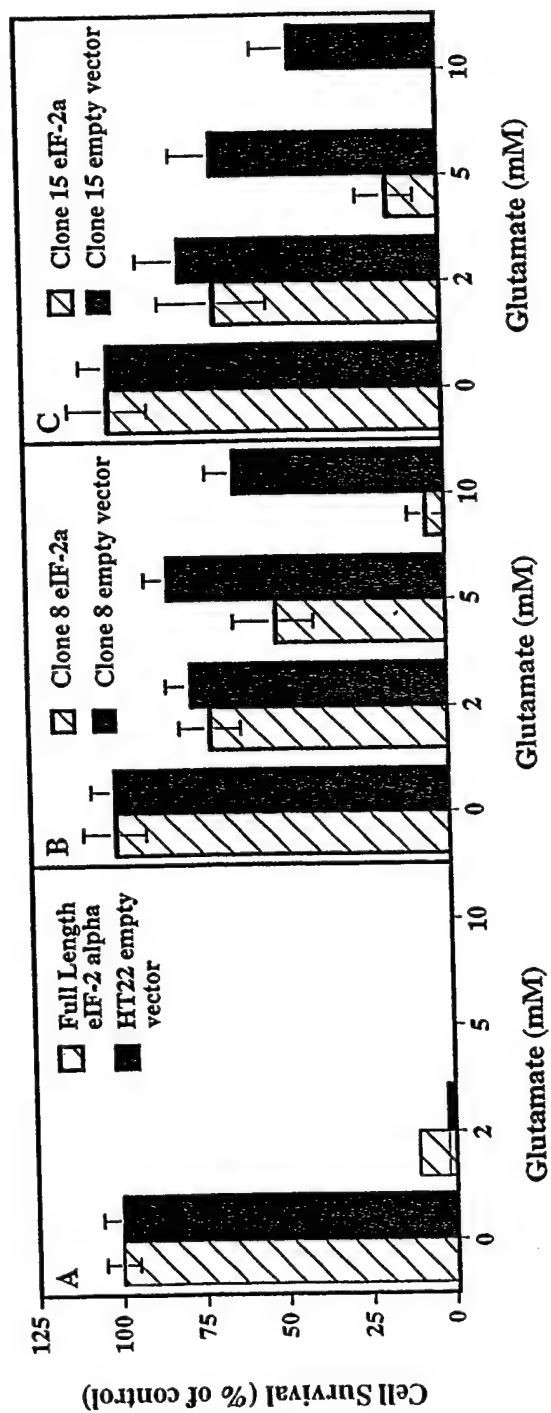
(A)  $\gamma$ GCS protein and mRNA expression was measured in wild-type HT22 cells (lane 1), cells infected with the empty vector (pCLBAGE) (lane 2), and the resistant clones 8 (lane 3) and 15 (lane 4) by western and northern blot analysis, respectfully.

(B) Resistant clones 8 and 15 were infected with wild-type eIF2 $\alpha$ , or the wild-type clone infected with eIF2 $\alpha$ , S51A or S51D and the levels of  $\gamma$ GCS and actin determined by western blotting. Lanes 1. Resistant clone Cl8 plus eIF2 $\alpha$  ( $82 \pm 7\%$  of Cl8, lane 2); 3, Resistant clone 15 plus eIF2 $\alpha$  ( $61 \pm 5\%$  of Cl15, lane 4); 5, HT22 infected with empty pCLBAGE; 6, HT22 infected with S51A; 7, HT22 infected with S51D (a  $58 \pm 10\%$  increase relative to pCLBAGE; pCLBAGE and S51A were indistinguishable).

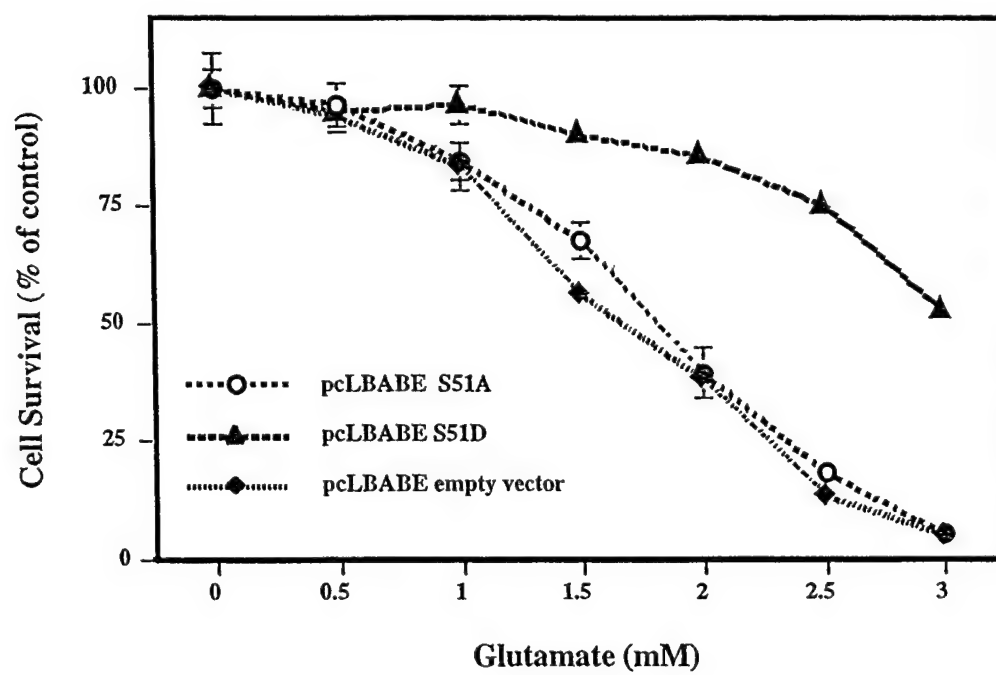
(C) The western blot (A) was analyzed using the program NIH image to determine the densities of each band. The densities were measured in 3 experiments, averaged, and normalized to pCLBAGE as 1.0. Actin served as loading controls and showed that there was an equal amount of protein in each lane. The experiment was repeated 4 times. Northern blots were analyzed both by NIH image and quantitated on a phosphoimager. The ratio of the catalytic subunit of  $\gamma$ GCS to actin is presented normalized to pCLBAGE as 1.0. The results were confirmed by RT-PCR analysis (data not shown).

(D) Proteolytic breakdown of  $\gamma$ GCS and P27 in wild-type and resistant cells. HT22 cells and resistant clone 15 were treated with 100 $\mu$ g/ml cycloheximide and the amount of  $\gamma$ GCS and P27 quantitated by western blot at 2 hr intervals. The values are normalized to 0 time and the mean  $\pm$  SEM of triplicate experiments. Insert, western blots of  $\gamma$ GCS, wild-type cells (1);  $\gamma$ GCS, Cl 15 (2); P27, Cl15 (3). Lanes 1,2,3,4, and 5 are 0,2,4,6, and 8 hrs post cycloheximide.

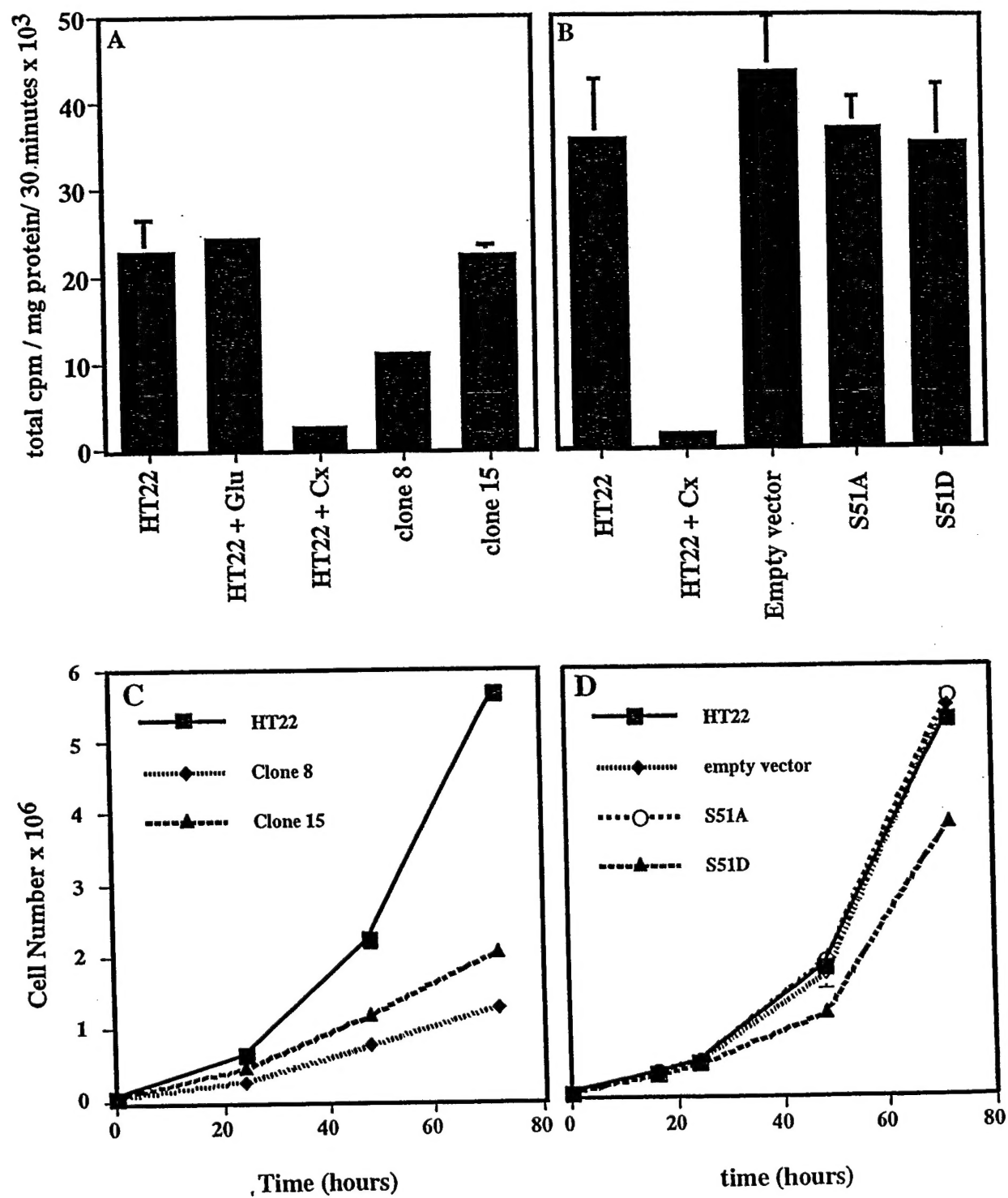




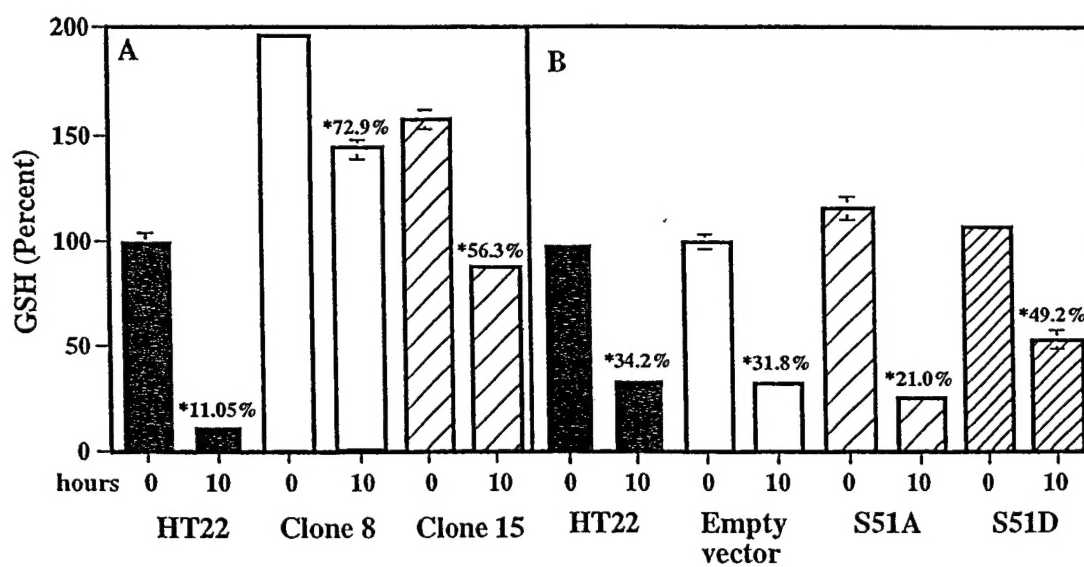
Tan et al.  
Figure 2



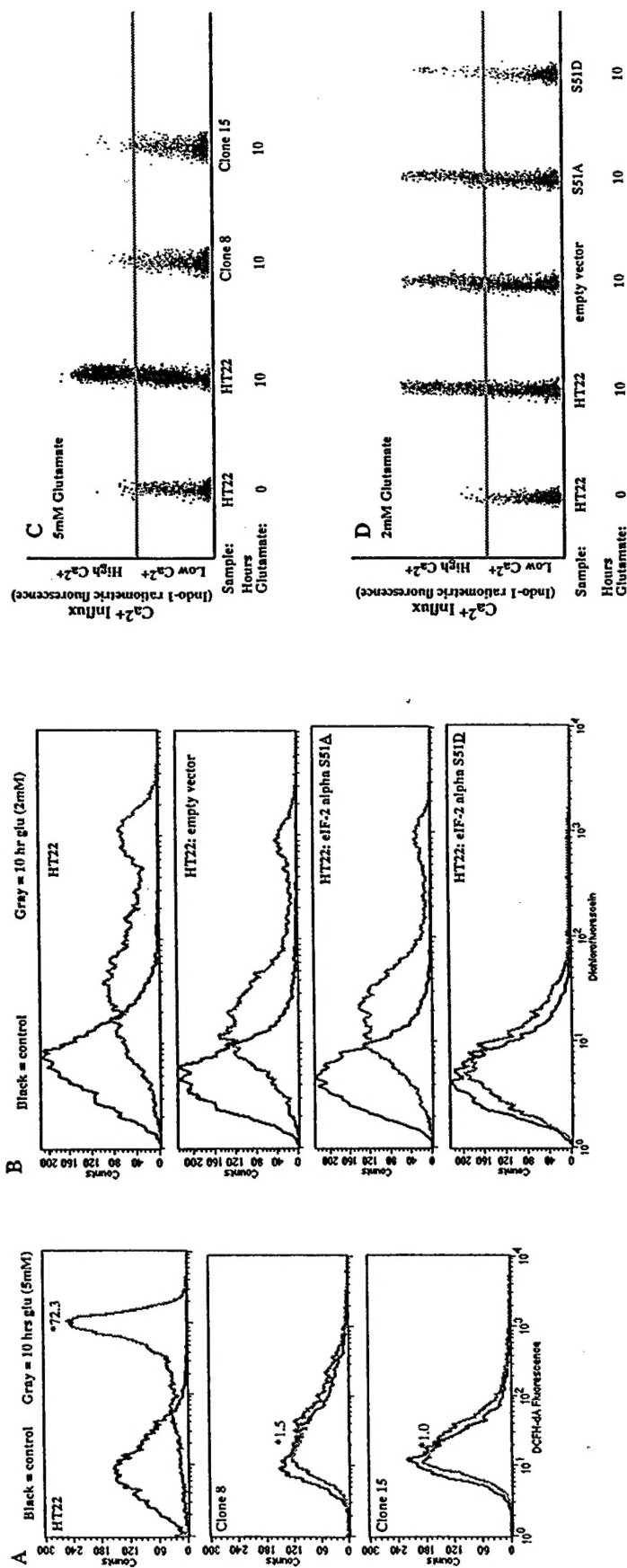
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Figure 3



Tan et al.  
Figure 4







Tan et al.  
Figure 6

